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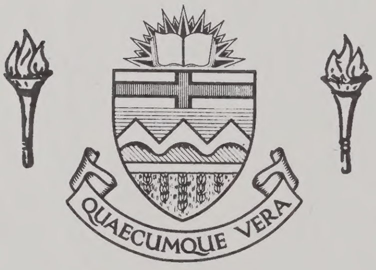

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STUDIES ON THE EFFECTS OF WHOLE-BODY GAMMA IRRADIATION ON SOME HEPATIC DRUG-METABOLIZING SYSTEMS IN MICE

by

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ABSTRACT

Groups of mice were exposed to whole-body gamma irradiation in doses ranging from 300R to 1200R, and several hepatic oxidative and reductive drug-metabolizing systems were examined at different post-irradiation intervals. The various degrees of stimulation in the *in vitro* metabolism of aniline, aminopyrine and p-nitrobenzoic acid which were noted in the early post-irradiation periods after whole-body exposure to 300R, 600R, 950R or 1200R were followed by a decline after the third day. Subsequent to the initial increases observed in the mice receiving 300R or 600R, the activities of aniline p-hydroxylase, aminopyrine N-demethylase and p-nitrobenzoic acid reductase sustained a period of inhibition, followed by recovery on the eleventh or fifteenth day respectively. Exposure to 950R or 1200R resulted in severe impairment of *in vitro* hepatic drug metabolism from the third day continually until death of the animals.

The protein content of liver microsomes was generally elevated on the third post-irradiation day, followed by a decline to normal or slightly subnormal level on day 5. Except for decreases observed on the seventh post-irradiation day in the mice receiving 950R and 1200R, the microsomal protein content remained at control values for the rest of the experimental period.

300R and 600R caused a cyclic pattern in microsomal cytochrome P-450 levels; i.e., an initial increase followed

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by a decline and subsequent return to normal. Hepatic cytochrome P-450 was depressed initially by 1200R and after the third day following 950R. The reductions in cytochrome P-450 were closely paralleled by the activities of the drug-metabolizing enzymes after the third or fifth post-irradiation days.

Stress-induced increases in the activities of drug-metabolizing enzymes, microsomal protein and cytochrome P-450 levels were observed in mice exposed to a cold environment. Similarities were noted in the initial responses of the hepatic detoxication systems following exposure to radiation or cold. It was suggested that the stimulation in the *in vitro* drug metabolism which was observed early after irradiation may have been caused by a radiation-induced stress reaction. Radiation injury to hepatic drug detoxication was evidenced by severe impairment in drug-metabolizing activity and in microsomal cytochrome P-450 during the later post-irradiation stages.

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I. INTRODUCTION

During the past fifteen years, a vast accumulation of scientific data has indicated that a large variety of drugs and foreign chemicals are metabolized by enzyme systems located in the endoplasmic reticulum of the liver. The importance of these systems has received increased recognition in therapeutics and toxicology and has provided an impetus for numerous current studies concerning the morphology of hepatic endoplasmic reticulum as well as the mechanisms for regulation and action of its enzymes.

Reports from numerous laboratories in recent years have shown that the activity of drug-metabolizing enzymes in liver microsomes can be influenced markedly by a wide range of factors including: the administration of hormones and various foreign compounds, age, sex, strain, and nutritional status of the animal as well as some unphysiological and pathological conditions (1).

Although the term "drug metabolism" is generally associated with the biotransformation of medicinal agents by the body, it is apparent that a large number of non-medical foreign compounds are also ingested daily by man. These chemicals include food and cosmetic additives, agricultural chemical residues, pesticides and an ever increasing array of contaminants of the environment. It is known that as a protection against the harmful effects of these substances, the body possesses a biochemical defense mechanism via hepatic detoxication reactions. However, it is important

to understand how this defense system works and what its limitations are.

With the advent of the nuclear era, mankind is confronted with increasing utilization of radiation in medicine, research and industry. Reports that irradiation had affected the development and activity of certain microsomal enzymes (2) suggest that exposure to ionizing radiation may also be added to the expanding list of factors which can modify hepatic detoxication systems.

It was the purpose of this investigation to study the effects of whole-body irradiation on some hepatic oxidative and reductive pathways in mice. Preliminary work immediately indicated that two crucial factors influencing the radiation-induced changes in hepatic metabolic activity were: the initial dose of radiation, and the time interval after irradiation. This study was then expanded to include radiation levels in both the lethal and sublethal range and post-irradiation intervals up to 21 days where survival of the animals permitted. Besides measuring the activities of hepatic aniline p-hydroxylase, aminopyrine N-demethylase and p-nitrobenzoic acid reductase, the protein content of the microsomal fraction was also determined in an effort to relate enzyme activity with protein levels.

Recent advances in the elucidation of some of the mechanisms involved in the electron transport system of microsomal drug-metabolizing pathways confirm that cytochrome P-450 is a very vital component of these reactions. Therefore,

liver microsomes were assayed for cytochrome P-450 to ascertain if changes in hepatic detoxication capacity could be reflected by P-450 levels following irradiation.

In order to observe whether the responses of the hepatic microsomal systems to ionizing radiation were similar to non-specific stress reactions, groups of mice were exposed to acute cold conditions. The activities of oxidative and reductive drug-metabolizing enzymes, as well as protein and cytochrome P-450 levels in the liver microsomes were than determined for comparison with the results from the irradiated animals.

II. SURVEY OF THE LITERATURE

1. Historical

The discovery that hippuric acid was synthesized in the mammalian organism from ingested benzoic acid was one of the earliest known biochemical reactions of the type which are now classified as "detoxication mechanisms". Hippuric acid biosynthesis was conclusively established by Keller in 1842 after many years of confusion of this conjugate with its precursor, benzoic acid (3). The next metabolic pathway to be discovered was the biological oxidation of benzene into phenol (1867), followed by demonstrations of ethereal sulfate conjugations (1876) and glucuronide conjugation (1879) as well as mercapturic acid synthesis (3). During the period between 1875 and 1895, a large majority of the commonly known detoxication reactions were ascertained. Studies of the metabolism of foreign compounds closely followed the development of organic chemistry and as new compounds were synthesized their toxicity and fate in animals were investigated. Williams, in his treatise "Detoxication Mechanisms" (3), has outlined the discoveries of biochemical pathways for a vast number of organic compounds, and this volume serves as an excellent source of information on the metabolism of drugs and chemicals by providing encyclopedic treatment of the subject. It was of significance that in the elucidation of these biotransformations, the liver was shown to be the major organ of involvement. As noted in the above text, the metabolism of foreign substances was studied mainly by analysis of urine

for metabolites. At first, these various metabolic pathways were regarded as merely biochemical reactions of the foreign compounds and it was not until the turn of the century that their role in reducing the toxicity of synthetic compounds became recognized.

An important contribution to the study of drug metabolism was made by Claude in 1938, who applied the newly developed method of high speed centrifugation to the separation of cellular components (4). Claude observed the presence of a slowly sedimenting fraction composed of lipid, protein and ribonucleic acid (5). He called this fraction "small particles" and coined the name "microsomes", meaning small bodies. From these early studies, a wealth of experimental findings related to the role of enzymatic metabolism of foreign compounds has unfolded.

One of the most notable recent advances was the observation by Brodie and his co-workers that the enzymes effecting many of these metabolic transformations were located in the microsomes of the liver cells (6). Subsequent research has resulted in a much deeper understanding of the mechanisms of many detoxication reactions and has led to a concept that these are special enzymatic processes active in the neutralization and elimination of toxic substances and drugs.
More recently, evidence has also been provided that steroid hormones can serve as normal body substrates for drug-metabolizing enzymes in the liver microsomes (7).

2. Biochemical Reactions Undergone by Drugs

In spite of the diversity of chemicals to which the mammalian system is exposed, numerous studies have shown that a vast majority of drugs and xenobiotics are metabolized by relatively few reactions. These reactions can be classified as: oxidations, reductions, hydrolyses and conjugations (8). A number of volumes (3, 9, 10) and comprehensive review articles (11, 12) have presented extensive data about these biotransformations.

Conjugations are biosyntheses in which foreign compounds or their metabolites combine with readily-available endogenous substrates to form glucuronides, ethereal sulfates, mercapturic acids, amino acid conjugates, acetylated amines or methylated compounds. Conjugation is achieved by addition to a functional group of the foreign compound and generally results in the formation of a more polar molecule which can be more readily excreted from the body (9).

Hydrolytic detoxication is effected by a number of enzymes which split their substrates by the addition of water. De-esterification and deamidation reactions are common examples of this pathway (11).

The oxidative systems for metabolism of drugs include a number of reactions in which oxygen, as a hydroxyl moiety, participates in one or more of the steps leading to the formation of the final metabolite (13). Oxidative reactions mediated by the mammalian microsomal system include: (a) hydroxylation of aromatic rings; (b) hydroxylation of

alkyl carbon chains; (c) deamination; (d) O-dealkylation; (e) N-dealkylation; (f) N-oxidation; and (g) sulfoxidation (11, 12).

The animal body also contains enzymes which catalyze the reduction of foreign compounds. A variety of aromatic nitro compounds such as nitrobenzene, p-nitrobenzoic acid and chloramphenicol, as well as azo compounds, are reduced to the corresponding amines (11).

3. Localization of Drug-Metabolizing Enzyme Systems

The involvement of the liver in the biotransformations of drugs and synthetic chemicals has been recognized for a long time (3, 11). However, drug metabolizing enzyme systems have been found in other tissues as well. Thus, conjugation reactions have also been reported in kidney, gastro-intestinal mucosa and skin, while blood plasma has been shown to contain hydrolytic esterases and amidases (11, 12). It has been indicated that the enzymes responsible for the oxidation and reduction of drugs are located mainly in the liver, with only minor amounts occurring in other tissues such as kidney, lung, adrenal and gastro-intestinal tract (14).

Evidence has been presented that drug-metabolizing activity in the liver is localized in the endoplasmic reticulum (6). The endoplasmic reticulum of liver cells is a lipo-protein tubular network which extends from the cell wall throughout almost all regions of the cytoplasm (15). The reticulum consists of two major components: a rough-

surfaced form comprised of lipoid tubules studded with small dense particles called ribosomes, and a smooth-surfaced form in which the tubules are devoid of ribosomes (16). Upon homogenization, the network of tubules is fragmented to form small vesicles known as microsomes. Centrifugation of tissue homogenate at 10,000 x g for 10 minutes deposits the cell debris, mitochondria and nuclei. Further high speed centrifugation of the supernatant at 100,000 x g for one hour results in a pellet of microsomes and the second supernatant which is known as the soluble fraction (16). Thus the microsomal pellet consists of both rough and smooth-surfaced fragments of endoplasmic reticulum. Subfractionation of the total microsomal fraction into "rough" and "smooth" microsomes has been achieved by density gradient centrifugation (17, 18). Ribosomes were shown to play an essential role in the synthesis of protein, including enzymes (14, 15, 19), whereas the drug-metabolizing enzymes were mainly associated with the smooth-surfaced microsomes (17, 20). Numerous research projects have been devoted to the distribution of drug-metabolizing enzymes in the rough and smooth microsomes (17, 21, 22, 23, 24). In a recent symposium, Fouts and Gram (25) concluded that a majority of NADPH-dependent enzymes were relatively more concentrated in smooth than in rough-surfaced microsomes, but that somewhat varied results may be obtained depending on the method of fractionation and the particular pathways studied. It has not been resolved whether the enzyme

systems are localized in the lumen of the endoplasmic reticulum or in the lipoid membranes, although some evidence points to the latter (14).

4. Drug-Oxidation in Hepatic Microsomal Systems

a. Requirements for Microsomal Drug-Oxidation

Axelrod (26, 27) and Brodie and his co-workers (6) have established that in the mammal, the primary site of drug-metabolizing activity is in the liver microsomes, with some necessary co-factors for the various reactions being found in the soluble fraction. Reports from numerous investigations noted that oxygen and reduced nicotinamide adenine dinucleotide phosphate (NADPH) were necessary for the activity of the oxidative enzymes (6, 26, 27, 28). The requirement for microsomes as well as soluble fraction in the *in vitro* incubation media suggested that glucose-6-phosphate together with glucose-6-phosphate dehydrogenase in the soluble fraction served to maintain NADP in a reduced form (6, 28). Direct evidence that NADPH could replace the glucose-6-phosphate plus soluble fraction was obtained from experiments which showed that chemically prepared NADPH added directly to washed microsomes promoted the metabolism of various drugs (6, 29, 30). Addition of NADH instead of NADPH greatly reduced the amount of metabolite formed, thus indicating the specificity of the enzyme system for NADPH (6, 27). Nicotinamide, when added to drug incubation mixtures, was found to enhance the *in vitro* metabolism (6,

12, 27) by protecting NADP from destruction by any NADPase present in microsomal preparations (31). Magnesium ions were shown to activate the NADPH generating system by increasing the activity of glucose-6-phosphate dehydrogenase (27, 30).

The dependence of the microsomal oxidative pathway on the availability of oxygen was illustrated when incubation under anaerobic conditions prevented the *in vitro* metabolism of amphetamine (26). It was further established, by utilizing ^{18}O, that oxygen incorporated into the substrate originated from the atmosphere rather than from the water molecules (32, 33).

b. Mechanisms of Drug-Oxidation

Mason (32) introduced the term "mixed function oxidase" for enzymes which are involved in the introduction of an atom of molecular oxygen into the substrate molecule concomitant with the oxidation of reduced NADP. It was suggested by Gillette (11, 14) that NADPH served to reduce a component "A" in microsomes that reacted with oxygen to form an "active oxygen" intermediate. The "active oxygen" was thought to oxidize the substrates by a group of non-specific enzymes in the microsomes. The proposed mechanism was formulated as:

(1) $\text{NADPH} + \text{A} + \text{H}^+ \longrightarrow \text{AH}_2 + \text{NADP}^+$

(2) $\text{AH}_2 + \text{O}_2 \longrightarrow$ "active oxygen"

(3) "active oxygen" + drug $\longrightarrow$ $\text{drug-OH} + \text{A} + \text{H}_2\text{O}$

According to this view, most of the reactions catalyzed by

microsomal NADPH-dependent oxidative enzymes could be envisioned as hydroxylation reactions (13, 14).

Important contributions were made to the advancement of mechanisms concerning oxygen activation and the reactions associated with microsomal electron transport by the discovery (34, 35) and characterization (36, 37) of cytochrome P-450 in liver microsomes. Subsequent investigations showed that cytochrome P-450 was an essential component of the mixed function oxidases involved in the hydroxylation of steroids and many foreign compounds (38, 39). As a result of intensive research into the relation between cytochrome P-450 and microsomal drug metabolism, the formerly proposed mechanism of oxygen activation was revised. A currently accepted scheme (24, 40) is presented in Figure 1. According to Gillette (41), substrates react with the oxidized form of cytochrome P-450. This cytochrome P-450 - substrate complex is then reduced, either directly by NADPH cytochrome c reductase (41, 42), which is an FAD-containing flavoprotein, or indirectly through an intermediate non-heme iron protein, as yet unidentified and called Fe_x (42, 43). The reduced cytochrome P-450 - substrate complex then reacts with atmospheric oxygen to form an O_2-cytochrome P-450 - substrate complex which subsequently decomposes with the concomitant oxidation of both the drug and the P-450 (44). Some researchers measure the total amount of cytochrome P-450 which is reducible by NADPH, either via NADPH cytochrome c reductase or Fe_x, and refer to this process collectively as "NADPH

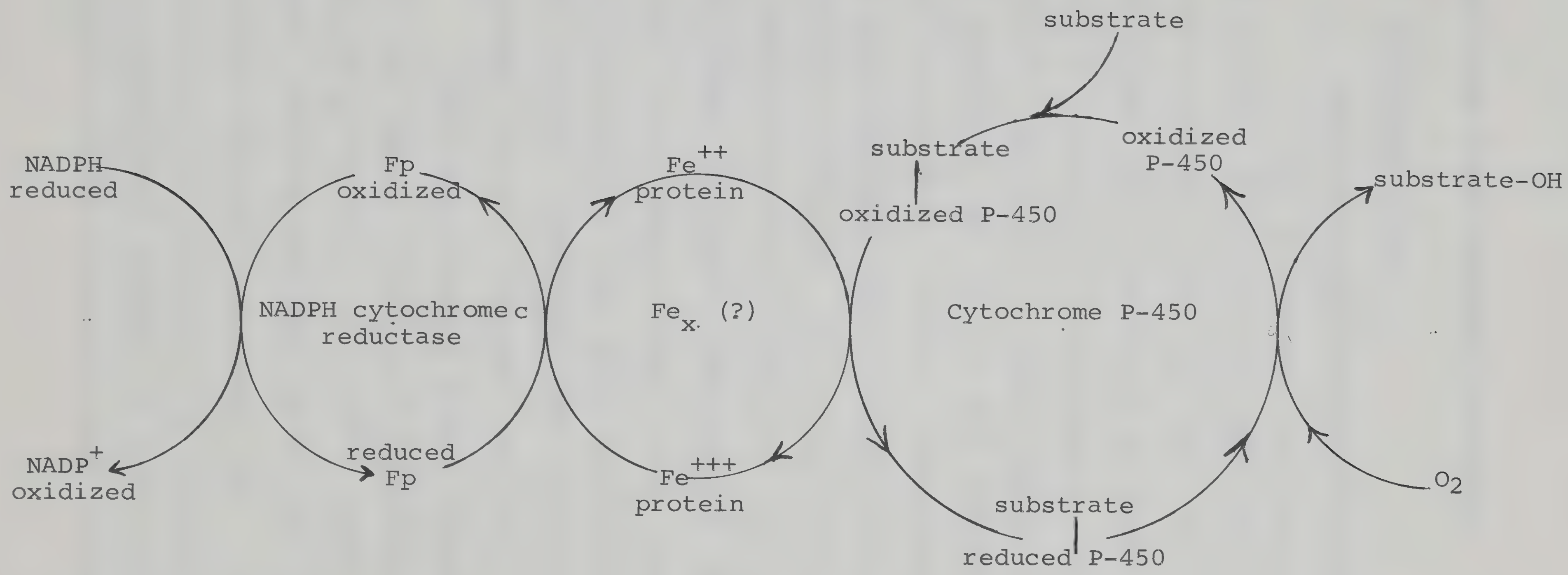


Figure 1. Sequence of reactions involved in the mixed-function oxidases of hepatic microsomes

cytochrome P-450 reductase" activity.

5. Cytochrome P-450

a. Distribution and Characterization

In 1958, Klingenberg (34) and Garfinkel (35) reported the presence of a carbon monoxide-binding hemoprotein in liver microsomes. Omura and Sato (36, 37) indicated that this pigment was a cytochrome and named it cytochrome P-450 from the position of the absorption peak of the carbon monoxide complex of the reduced hemoprotein. Estabrook and his associates (38, 39, 40) have demonstrated that cytochrome P-450 functions as a terminal oxygen-activating enzyme for the NADPH-dependent mixed-function oxidase system of mammalian microsomes. Cytochrome P-450 has also been found in adrenal-cortico microsomes and mitochondria, where it participates in steroid hydroxylation (38, 40) and to a small extent in microsomes of other animal tissues including kidney, intestinal mucosa, lung, ovary and pituitary gland (45). More recently, cytochrome P-450 has been identified in the microsomal fraction of human liver (46).

The elucidation of precise molecular mechanisms involving oxygen activation by cytochrome P-450 has been hampered by the fact that P-450 is bound firmly to the endoplasmic reticulum membrane and has not yet been isolated in its native form from microsomes (47). Attempts at solubilizing the hemoprotein by treatment of liver microsomes with deoxycholate, steapsin or snake venom resulted in the conversion of the cytochrome P-450 to a metabolically inactive

form, the reduced CO complex of which had an absorption maximum at 420 mμ (36, 37, 38). Various organic compounds were also shown to effect the transformation of P-450 to P-420 (42, 45, 49, 50).

b. Interaction of Drugs and Cytochrome P-450

It has been reported that various drugs or other substrates react with hepatic microsomal cytochrome P-450 to give two characteristic kinds of spectral changes called "type I" and "type II" (51, 52, 53). Remmer and co-workers (51) and Imai and Sato (52) showed that drugs could be classified into two groups with respect to the difference spectra they produced when combined with microsomal hemoprotein. Type I spectral change was caused by a group of compounds such as aminopyrine, hexobarbital, phenobarbital or benzphetamine and was characterized by the appearance of a trough at 420 mμ and an absorption band at 385 mμ. A second type of spectral change (type II) was obtained on the addition of substrates such as aniline, pyridine or DPEA (a metabolic inhibitor). In this case, an absorption peak appeared at about 420 mμ and a trough at 390 mμ. It was concluded that the spectral changes were indicative of substrate-cytochrome P-450 interactions and that type I and II binding could be explained on the basis of two binding sites on a single hemoprotein molecule with different drugs combining preferentially with one or the other site (51, 52, 54). Alternately, the existence of two separate hemoproteins which combine selectively with various drugs has also been

suggested (54, 55). The intensity of these spectral changes has been shown to be dependent on the concentration of both the substrate and the microsomal cytochrome P-450 (52, 54). Remmer et al. (53) found that the rate of metabolism of aminopyrine and hexobarbital was reflected by the magnitude of the spectral-induced changes. Some correlation was observed between increases in the rate of N-demethylation of benzphetamine or p-hydroxylation of aniline and in the increases in the respective type I or II difference spectra after phenobarbital treatment of rats (51, 56, 57). The aniline-induced difference spectrum (type II) as well as microsomal aniline p-hydroxylation activity was reported to be increased after induction by phenobarbital or methylcholanthrene; however, the metabolism of hexobarbital and associated spectral change (type I) was stimulated only by phenobarbital, but depressed by methylcholanthrene (58).

c. Microsomal Cytochrome P-450 and Drug Metabolizing Activity

Following the establishment that cytochrome P-450 functions as a terminal oxidase in hepatic microsomal reactions, numerous investigations have reported that alterations in microsomal drug-metabolizing activity could be correlated with changes in the content of cytochrome P-450 and also, in some instances, with changes in cytochrome P-450 reductase. Activities of aminopyrine N-demethylase paralleled the levels of cytochrome P-450 and NADPH cytochrome c reductase during phenobarbital enzyme induction as well as during

enzyme inhibition by actinomycin D (21, 59). Gram *et al.* (60) have shown that the stimulatory effect of phenobarbital on hepatic p-nitroanisole demethylase and aniline p-hydroxylase was associated with an increase in the content of cytochrome P-450.

Increases in the rates of drug metabolism after phenobarbital induction and subsequent declines after withdrawal of the inducing agent have been shown to parallel closely with the increases or decreases in the level of microsomal cytochrome P-450 (19, 20, 53, 61, 62). Several workers have shown that the oxidation of drugs by microsomal enzymes was related not only to the content of P-450 but also to the activity of NADPH cytochrome P-450 reductase (63, 64, 65, 66).

Impairment in the activity of NADPH-dependent enzymes in liver microsomes by a variety of factors has been associated with decreased amounts of cytochrome P-450. Administration of carbon tetrachloride, an hepatotoxic agent, resulted in inhibition of drug-metabolizing activity, as well as in a reduction of cytochrome P-450 in liver microsomes (67, 68, 69, 70). A decrease in *in vitro* drug metabolism by liver microsomal enzymes, together with a reduced content of cytochrome P-450, was noted in tumor-bearing rats (71, 72, 73) and in animals pretreated with morphine (74) or the herbicide aminotriazole (75, 76).

Administration of polycyclic hydrocarbons such as benzpyrene or 3-methylcholanthrene had resulted in a

stimulation of certain hepatic microsomal oxidative pathways, together with an increase in cytochrome P-450 (77) as well as in the formation of a new hemoprotein called cytochrome P_1-450, P-446 or P-448 (55, 78, 79, 80, 81).

The voluminous reports concerning microsomal drug metabolism and cytochrome P-450 are a reflection of the intense, current interest in this area. A more exhaustive research of this literature is outside the scope of this thesis and the reader is directed to a recent symposium on "Microsomes and Drug Oxidations" (82) as well as several reviews on the subject (14, 83).

6. Microsomal Protein and Drug Metabolism

The preceding pages have referred to the importance of the levels of cytochrome P-450 and other components of the NADPH-dependent electron transport chain in the drug-metabolizing activity of liver microsomes. However, a number of reports have indicated that alterations in microsomal drug metabolism were also associated with changes in the protein content of the microsomal fraction. The increases in hepatic drug detoxication seen when animals were treated with various drugs, hormones, insecticides or carcinogens has been referred to as "enzyme induction" and this rise in activity appeared to represent an increased concentration of enzyme protein (83). Phenobarbital, one of the most commonly used enzyme inducers, has been shown to cause an increase in: (a) the metabolism of a large

variety of compounds by liver microsomes; (b) microsomal cytochrome P-450; and (c) microsomal protein content (21, 59, 60, 84, 85). The finding that inhibitors of protein synthesis such as ethionine, puromycin or actinomycin D were able to block the induction of drug-metabolizing enzyme activity by phenobarbital or 3-methylcholanthrene provided evidence that enzyme induction in microsomes was due to an increase in protein (83). It has been suggested that phenobarbital increased the total protein level in liver microsomes by stimulating the rate of synthesis and decreasing the breakdown of microsomal protein (85, 86).

The inhibition of microsomal drug-metabolizing activity which was produced by a low-protein diet was paralleled by a reduction not only in cytochrome P-450, but in microsomal protein content as well (87, 88).

Several experiments have shown that some changes in drug-metabolizing activity may not necessarily be accompanied by alterations in microsomal protein content. A decrease in oxidative and reductive metabolic activities was reported in the hepatic microsomes of tumor bearing rats, although there was no significant change in microsomal protein content, but a marked decrease in cytochrome P-450 (71, 72, 89). The liver microsomal protein levels in young and old rats were found to be almost the same, yet the activities of hexobarbital hydroxylation, aminopyrine N-demethylation, strychnine oxidation, aniline hydroxylation and p-nitrobenzoic acid reduction were all greater in the young rats,

as were the levels of cytochrome P-450 and NADPH cytochrome c reductase (90).

7. Factors Which Influence Hepatic Drug Metabolism

The activities of drug-metabolizing enzymes in liver microsomes have been reported to be markedly affected by a wide spectrum of pharmacological, physiological and environmental conditions and agents. Several review articles, supplemented with numerous references, have dealt extensively with many of these parameters (1, 11, 12, 83). Therefore, the various factors will only be outlined here with emphasis on the ones that may be pertinent to the experimental portion of this thesis.

a. Species

Within the mammalian group, wide qualitative as well as quantitative differences are seen in relation to drug-metabolizing activity. For example, cats do not readily form glucuronides, dogs are unable to acetylate aromatic amines and guinea pigs do not appreciably acetylate S-cysteinyl derivatives (11). Furthermore, amphetamine is mainly deaminated in rabbits, but hydroxylated in dogs; aniline is hydroxylated primarily in the para position in rabbits and rats but in the ortho position in cats (11, 12).

Most species differences, however, are accounted for by variations in the rates of metabolism of drugs. Aminopyrine N-demethylation and aniline p-hydroxylation were found to proceed about three times as fast in mouse liver as in rat

liver (61, 91) and p-nitrobenzoic acid reductase activity was reported to be as much as ten times higher in mice than in rats (92). Some of the species differences in aminopyrine and aniline metabolism have recently been ascribed to cytochrome P-450 content (61).

Knowledge of species differences in drug metabolism has been shown to be of considerable importance in extrapolating pharmacological data obtained from animals to man (1, 93).

b. Strain

Quinn *et al.* (91) observed pronounced differences in the rate of antipyrine metabolism by eight different strains of rats. A three-fold variability in response to hexobarbital among members of several strains of mice has been reported, although a marked uniformity existed among individuals of a highly inbred strain (1). Alterations in drug-detoxication in certain strains have been ascribed to deficiencies in particular enzyme systems (11).

c. Sex

Adult male rats metabolize many drugs and other foreign compounds at faster rates than female rats (11). This enhancement of activity of certain hepatic microsomal enzymes appeared to be due to sex hormones since: (a) the difference was obvious only after puberty; (b) castration of the males abolished the high enzyme activity; and (c) administration of androgens to female rats increased the activity of the microsomal enzyme to the level of the males (12). Sex

differences in drug metabolism have recently been related to the magnitude of cytochrome P-450 - substrate binding in liver microsomes from male and female rats (94).

Sex variation in drug metabolism appears to be restricted to the rat, since no sex differences in microsomal metabolism were observed in mice, guinea pigs, rabbits or dogs (11).

d. Age

Newborn mice, rats, guinea pigs and rabbits were noted to lack the microsomal enzymes, including cytochrome P-450 (61), which are necessary for the metabolism of drugs and foreign compounds (1, 11, 12). These enzyme systems began to develop during the first few postnatal days and increased rapidly within the first week. Adult levels were reached in about four or five weeks (12, 61).

The therapeutic implications involving the recognition of deficient drug-metabolizing systems in infants were emphasized by the toxic reactions caused by administration of chloramphenicol to premature or neonatal babies (95).

Kato and Tanaka (90, 96) have shown that drug-metabolizing activity declines with old age. The metabolism of drugs as measured by *in vitro* experiments using liver microsomes (90) or *in vivo* studies measuring zoxazolamine paralysis, pentobarbital narcosis or strychnine toxicity (96) all indicated that the rate of metabolism in 600 day old rats was significantly less than in young rats (100 days old). Further investigation established that the impaired

metabolism in old rats was associated with low levels of NADPH-electron transport components (90).

e. Stimulation by Foreign Compounds

The activation of the metabolism of synthetic chemicals by the administration of other foreign compounds, such as drugs, pesticides and polycyclic hydrocarbons, is well known and has been studied extensively because of the fundamental association of this phenomenon with enzyme induction, drug synergism and carcinogenesis. A comprehensive review (379 references) of the subject has recently been presented by Conney (83). Foreign compounds evoke stimulation of microsomal activity only when administered to the living animal. When added to incubation mixtures, they do not enhance enzyme activity (12, 83). Moreover, maximal acceleration of drug biotransformation is seldom seen within 24 hours after administration, but at a later time interval (11). Such activation of microsomal enzymes has been demonstrated in many species, including man, rat, mouse, rabbit, guinea pig, hamster and dog (1, 83).

More than 200 drugs, insecticides, carcinogens and other chemicals are known to stimulate the activity of drug-metabolizing enzymes in liver microsomes. These inducer compounds are extremely diverse pharmacologically and there appears to be no apparent structure-activity relationship in their ability to increase enzyme activity (83).

Enzyme-inducers are of at least two general types as exemplified by phenobarbital and 3-methylcholanthrene (12,

83). Many compounds are like phenobarbital in that they stimulate a large number of various pathways of metabolism by liver microsomes, including oxidation and reduction reactions as well as glucuronide conjugation and de-esterification (83). The increase in activity of the hepatic microsomal system brought about by phenobarbital pretreatment has been associated with increased content in cytochrome P-450 (19, 60, 61, 62), proliferation of the smooth endoplasmic reticulum (20) and elevated levels of microsomal protein caused by enhanced enzyme synthesis and a decreased rate of enzyme degradation (85, 86).

In contrast, polycyclic aromatic hydrocarbons typified by 3-methylcholanthrene and 3,4-benzpyrene stimulate only a limited group of reactions (83). Although these compounds also cause an elevation in microsomal protein and cytochrome P-450, it has recently been suggested that polycyclic hydrocarbons stimulate microsomal drug-metabolizing activity mainly by eliciting the formation of a new hemoprotein (55, 78, 79, 80).

f. Inhibition by Foreign Compounds

A number of drugs and other foreign compounds are known to inhibit microsomal drug-metabolizing enzymes through several different mechanisms. SKF 525-A (β-diethylaminoethyl diphenylpropyl acetate) has been shown to prolong the action of a diversity of drugs by inhibiting their rate of biotransformation *in vivo* (97). As well, addition of SKF 525-A to *in vitro* incubation mixtures resulted in interference

with the metabolism of the drugs by liver homogenates (98, 99). Other inhibitors include such compounds as DPEA, Lilly 18947, iproniazid, imipramine and glutethimide (1, 11, 14). Kato *et al.* (100) reported the inhibitory effects of over 40 different compounds on the oxidative microsomal systems of rat liver.

Since various inhibitors affected different microsomal enzymes, it was then suggested that a number of inhibitory mechanisms existed, such as interference with NADPH-generating systems, interference with reduction of cytochrome P-450, inactivation of P-450, blockade of the transfer of active oxygen from P-450 to the drug substrate and others, as yet unknown (14). Drugs such as ethionine, puromycin and actinomycin D inhibited protein synthesis, thus preventing stimulation of drug-metabolizing activity by enzyme inducers (83).

Administration of various hepatotoxic agents such as yellow phosphorous, 2-acetylaminofluorene or carbon tetrachloride had also resulted in a marked inhibition of drug-metabolizing activity in liver microsomes (1). Recently, a number of reports have indicated that carbon tetrachloride pretreatment impaired hepatic microsomal detoxication by depressing the level of cytochrome P-450 (67, 68, 69, 70).

g. Hormones

i. Thyroid

The effects of thyroid hormone upon the drug-metabolizing activities of liver microsomes have been shown

to be species dependent and in rats, sex dependent. In the female rat, thyroxine caused an increase in the metabolism of aminopyrine, hexobarbital, aniline and p-nitrobenzoic acid, whereas in the male only aniline p-hydroxylation was increased, while aminopyrine N-demethylation and hexobarbital hydroxylation were depressed, and p-nitrobenzoic acid reduction was unchanged (101). In mice, thyroid hormone inhibited the hepatic microsomal metabolism of aminopyrine, hexobarbital, aniline and p-nitrobenzoic acid. However, in rabbits, aminopyrine N-demethylase, aniline p-hydroxylase, and p-nitrobenzoic acid reductase were stimulated and hexobarbital hydroxylase remained unchanged after thyroxine treatment (102).

ii. Adrenal

It has been reported that adrenalectomy in rats caused an impairment of hexobarbital and aminopyrine metabolizing enzymes, but that the activity of these enzymes could be restored by administration of cortisone or prednisolone (11, 14, 83). Kato and Gillete (103) found, however, that adrenalectomy did not cause a decrease in aniline or zoxazolamine metabolism. More recently, Ichii and Yago (104) reported that decreases in aminopyrine N-demethylase activity after adrenalectomy were associated with reductions in microsomal P-450 content and that replacement therapy with cortisol partially elevated the cytochrome P-450 levels and restored N-demethylase activity. When prednisolone or hydrocortisone were added directly to *in vitro* incubation mixtures, aniline hydroxylation and aminopyrine N-demethylation

were inhibited due to competitive binding of the steroid hormones with cytochrome P-450 (105).

Acute or chronic administration of epinephrine and norepinephrine depressed the rate of *in vitro* microsomal metabolism of hexobarbital, aminopyrine and aniline in male rats (83) but had no apparent effect on drug metabolism in female rats (83, 103).

iii. Pituitary

A decrease in the hepatic metabolism of hexobarbital, aminopyrine and ethylmorphine was noted after rats were injected with a mixture of somatotropin, ACTH, and prolactin (106). Growth of a pituitary tumor which provided a continuous source of large amounts of rat corticotropin, prolactin and somatotropin was found to inhibit the microsomal metabolism of hexobarbital, aminopyrine and p-nitrobenzoic acid in the livers of the tumor bearing animals (89).

h. Pathologic and Disease Conditions

The activities of drug-metabolizing enzymes of liver microsomes were markedly decreased in male and female rats with abdominal carcinomas (71). The transformation of normal liver cells into cancerous cells by hematoma transplantation or by carcinogen induction resulted in severe reduction or complete loss of microsomal drug-metabolizing activity (1, 11, 83).

Livers from jaundiced animals have been shown to possess a markedly decreased ability to detoxify a variety of foreign compounds, presumably due to an increased hepatic

concentration of bile salts (1, 11, 12). Liver regeneration following partial hepatectomy was reported to be accompanied by decreases in glycogen content and microsomal activity, both of which were restored when regeneration was complete (1).

Alloxan-induced diabetes reduced the in vitro metabolism of hexobarbital, aminopyrine, codeine and chlorpromazine, but increased the hydroxylation of aniline (103) in male rats. These effects, which have been correlated to the levels of hepatic glycogen, were found to be reversed by treatment with insulin (1, 11).

i. Nutrition and Diet

The activity of the hepatic enzyme systems which metabolize foreign compounds can vary with the nutritional status of the animal. Considerable species differences in the alterations of these enzymes by starvation have been reported. In mice, Dixon et al. (108) noted that starvation decreased the in vitro metabolism of hexobarbital, chlorpromazine, aminopyrine and acetanilide, but that the reduction of p-nitrobenzoic acid and neoprontosil was either unaffected or even slightly increased. In female rats, starvation caused an enhancement of the activities of almost all the drug-metabolizing enzymes in liver microsomes (103). In contrast, starvation of male rats did not significantly affect the metabolism of nitroanisole, zoxozolamine, neoprontosil or p-aminobenzoic acid, but impaired the metabolism of aminopyrine, hexobarbital, pentobarbital and

morphine; and stimulated the metabolism of aniline, methylaniline and neotetrazolium (103, 109). More recently, Kato _et al._ (110) reported that starvation did not affect aminopyrine N-demethylating activity in mice, but that in rabbits, as in female rats, the activity of this enzyme system was considerably elevated.

Male rats which were maintained on a low protein diet for two weeks showed a decrease in aminopyrine N-demethylation, as well as significantly depressed levels of cytochrome P-450 and microsomal protein (88). Prolonged feeding with a low protein diet was associated with reduced activities of hepatic drug-metabolizing enzymes in both male and female rats, along with low levels of microsomal protein and cytochrome P-450 (87).

j. _Stress_

Adverse environmental conditions are known to influence some of the NADPH-dependent microsomal detoxication systems. The hydroxylation of acetanilide by rat liver microsomes was greatly increased by exposure of the animals to cold, while the N-demethylation of meperidine and methadone was decreased and the glucuronide conjugation of o-aminophenol was unaltered (111). Exposures to cold or cold plus high noise levels were reported to stimulate the hydroxylation of 2-naphthylamine in mice (112). Stitzel and Furner (113) have shown that exposure to cold for 6 or 12 hours caused an initial depression of ethylmorphine dealkylation, whereas a more prolonged exposure led to a significant increase in

the rate of ethylmorphine metabolism. The p-hydroxylation of aniline, however, increased immediately after cold treatment with no initial depression observed (113).

Rupe _et al._ (114), and Bousquet and co-workers (115), reported that short-term (2 - 5 hour) stress, such as hind limb ligation, shortened the duration of pharmacological response to drugs such as hexobarbital, pentobarbital, meprobamate and zoxazolamine. Direct evidence for stimulated drug metabolism in the stressed rats was obtained by measurements of drug blood levels and by _in vitro_ liver perfusion studies (116, 117). The ability of stress to decrease drug blood levels was shown to be dependent upon an intact pituitary-adrenal axis, since responses to limb ligation were blocked in adrenalectomized or hypophysectomized animals (116). Driever and Bousquet (117) have illustrated that actinomycin D could prevent the stimulation of pentobarbital metabolism which was produced by limb ligation, and suggested that increased protein synthesis may have been involved in the stress-mediated enzyme induction. Acute stress (limb ligation) and chronic stress (sodium amobarbital injections) were both shown to cause an increase in the urinary excretion of amobarbital and its metabolites (118).

The type of stress employed appears to be an important factor in the determination of the metabolic response that is elicited. Furner and Stitzel (119) have recently reported that long term cold stress (4 days at 4°C) had resulted in a depression of hexobarbital metabolism and

an elevation of ethylmorphine and aniline metabolism in rat liver microsomes. Furthermore, the metabolism of aniline and ethylmorphine could be stimulated even in adrenalectomized animals by long term cold exposure (119).

8. Effects of Ionizing Radiation on Hepatic Microsomal Enzyme Systems

Recent studies from various laboratories have suggested that exposure to ionizing radiation may be added to the list of factors that can influence microsomal enzyme activity. Hietbrink and DuBois (120, 121, 122) have demonstrated that whole-body irradiation of immature male rats with 200R or 400R severely inhibited the normal development of hepatic microsomal enzymes that metabolize organophosphates and hexobarbital, but did not affect the development of a reductase system responsible for metabolism of p-nitrobenzoic acid. Partial-body irradiation experiments revealed that shielding of the liver or testes did not prevent the inhibition of enzyme development, but that inhibition occurred when only the head was irradiated, indicating that it was due to an abscopal effect (123, 124). These same researchers also noted a lack of effects on hepatic enzyme activities in adult rats exposed to 400R of x-irradiation and concluded that radiation did not alter the ability of adult animals to detoxify foreign chemicals (120, 123). Since x-irradiation impaired the normal increase of oxidative microsomal enzymes in growing rats, but had no

effect on the development of reductase activity in the livers of these immature rats, Hietbrink and DuBois indicated that a selectivity in the action of x-rays on liver microsomal enzyme development may exist (120, 121).

Nair and Bau (125) observed that radiation doses in excess of 500R administered to the whole body, or above 1,000R to the head alone, depressed the activity of the hepatic microsomal enzyme system that metabolizes hexobarbital in adult male rats. *In utero* exposure of rats to low doses of x-irradiation produced, in the male offspring, an impairment in the development of hepatic microsomal enzyme systems (126, 127).

Some conflicting reports have appeared in the literature concerning the effects of irradiation on the process of enzyme induction. DuBois (124) observed that 400R of x-ray was not effective in blocking the phenobarbital induction of microsomal enzymes which were involved in organophosphate detoxication. Prenatal exposure of rats to x-irradiation did not suppress the phenobarbital induced increase of enzyme activity (127). Terayama and Takata (128) reported that 440R or 880R produced no impairment in methylcholanthrene induction of N-demethylase activity. Kato *et al.* (129) also found that radiation doses of 700R or 1400R failed to inhibit phenobarbital produced enzyme induction. In contrast, Ichii *et al.* (130) showed that the process of phenobarbital induction of aminopyrine N-demethylase was significantly inhibited by x-irradiation, and Pitot and

co-workers (131) reported that radiation could interfere with enzyme induction if the animals were irradiated within an hour after administration of the inducing agent.

The effects of irradiation on a number of conjugation reactions have been studied. 500R total-body irradiation was found to suppress conjugation of steroids in mouse liver (132) and the acetylation of sulfonamide in rat liver (133). Haley and Koste (134) noted a rise in glucuronide excretion on the first post-irradiation day, followed by a depression from the third day on. Local x-irradiation of the liver has been shown to cause an initial stimulation of *in vitro* o-aminophenol conjugation followed by a depression, and subsequently by an overcompensatory phase (135).

Some modifications of pharmacological activity have been noted in irradiated animals by several workers. The onset and duration of barbiturate hypnosis were found to be enhanced in irradiated animals (136, 137, 138). A study of the LD_{50} in mice of ten compounds representing four classes of drugs showed a tendency towards increased toxicity by the sixth post-irradiation day (139). In contrast, Doull (140) stated that irradiated animals exhibited few qualitative or quantitative changes in their response to therapeutic or toxic levels of a variety of commonly used drugs.

Several reports in the literature have indicated that irradiation with sublethal or lethal doses had no apparent effect on hepatic microsomal detoxication. Terayama and Takata (128) reported that N-demethylase

activity of normal rat liver was not altered even by 880R. Kato *et al.* (129) found no significant changes in the *in vitro* metabolism of pentobarbital or meprobamate in liver slices from rats irradiated with 750R or 1400R.

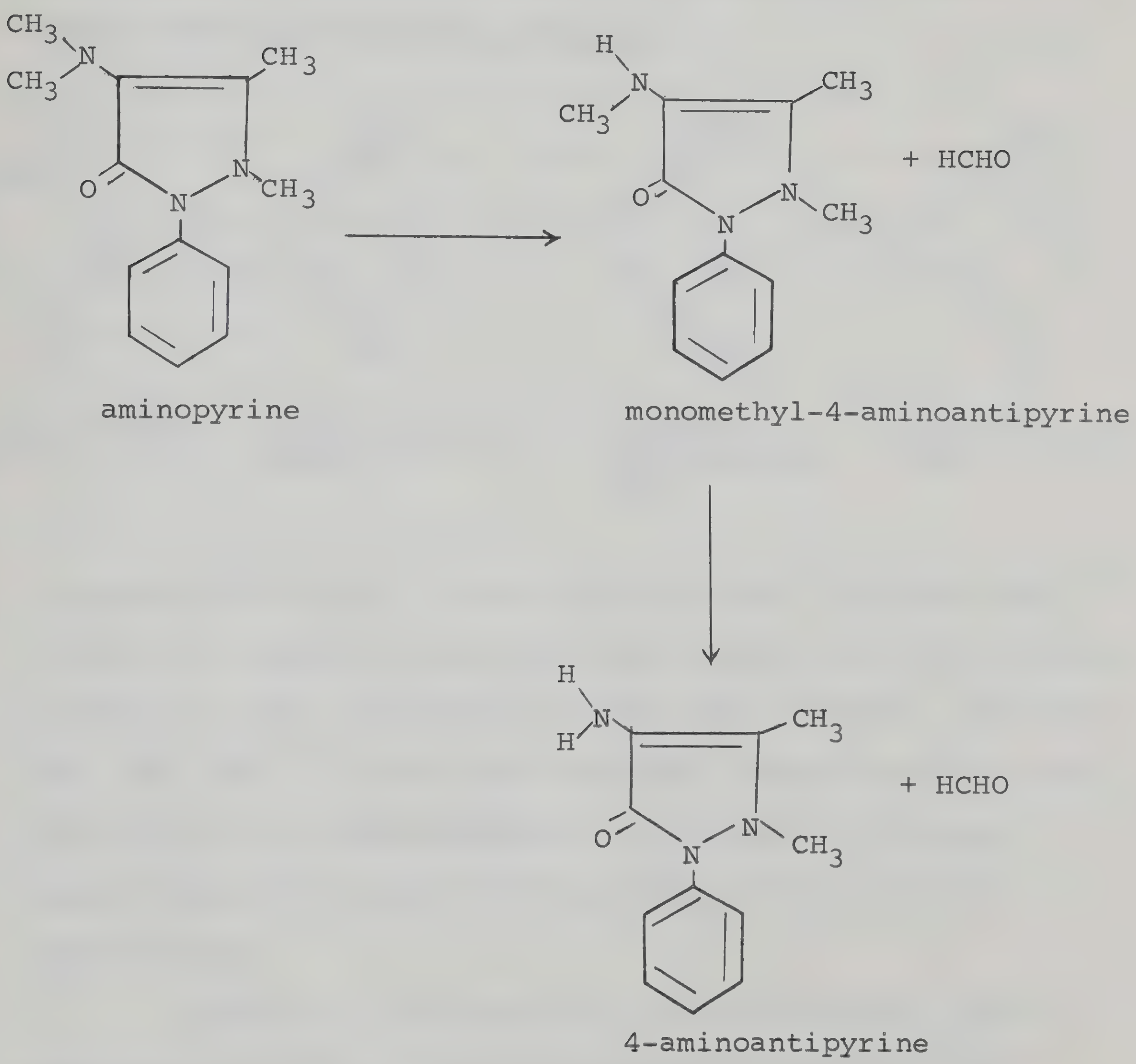
9. Metabolism of Aniline

It has been known since the work of Schmiedeberg and Müller in the late 1800's that aniline is oxidized in man and animals to p-aminophenol which is then excreted via the urine in a conjugated form (141). Parke and Williams (142) demonstrated the existence of a species variation in the ratio of para and ortho hydroxylation of aniline. In dogs and cats, o-aminophenol was the major oxidation product, whereas guinea pigs, hamsters, rabbits, rats and mice hydroxylated aniline almost exclusively in the para position. Incubation of aniline with hepatic rat or rabbit microsomes in the presence of NADPH and oxygen resulted in p-hydroxylation only (141).

10. Metabolism of Aminopyrine

Aminopyrine, also commonly known as pyramidon or amidopyrine, is 2,3-dimethyl-4-dimethylamino-5-oxo-1-phenylpyrazoline. Although aminopyrine is an effective antipyretic and analgesic, its therapeutic use has fallen into disfavor because it can produce agranulocytosis. In man, aminopyrine is almost completely metabolized at the rate of 10 to 30% per hour and excreted in the urine mainly as the acetyl derivative of 4-aminoantipyrine (3). The major metabolic route for the biotransformation of aminopyrine by liver

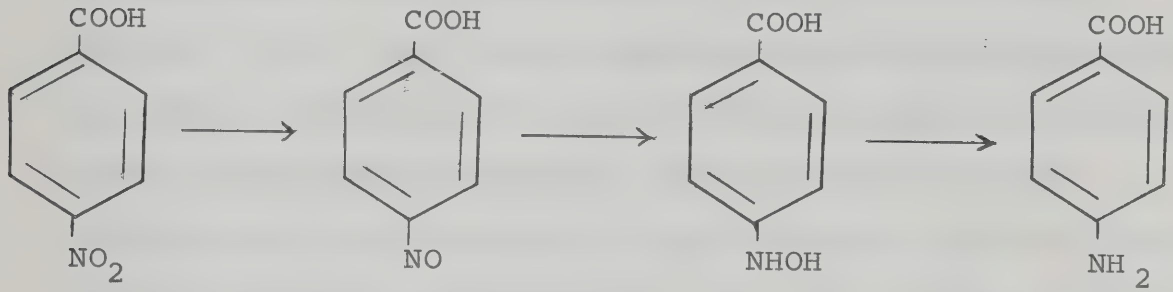
microsomes is demethylation as shown in the following sequence described by LaDu *et al.* (143):



This metabolic pathway has recently been confirmed by Gram *et al.* (144) who characterized the hepatic microsomal detoxication of aminopyrine in greater detail.

11. Reduction of p-Nitrobenzoic Acid

Fouts and Brodie (92) have reported that mammalian hepatic microsomal enzyme systems reduce p-nitrobenzoic acid to p-aminobenzoic acid through the intermediary formation of nitroso and hydroxylamine compounds:



p-nitrobenzoic acid

p-nitrosobenzoic acid

p-hydroxyaminobenzoic acid

p-aminobenzoic acid

A considerable amount of species difference has been shown to exist in hepatic nitro reductase systems, with the highest activity in mice, followed by guinea pigs, rabbits, rats and dogs (92, 145). Unlike most microsomal detoxication systems, the activity of nitroreductase was reported to be about equally divided between the microsomal and the soluble fraction (92).

Studies of the mechanism of p-nitrobenzoic acid reductase activity in liver microsomes showed that both NADP and NAD in the reduced form could act as hydrogen donors for the reduction of the nitro group, but that much greater activity was obtained using NADPH (92, 146). Kato et al. (147), however, reported that there are two different enzyme systems for the reduction of p-nitrobenzoate to p-hydroxy-

aminobenzoate in rat liver. NADPH-linked reduction of p-nitrobenzoate to p-hydroxyaminobenzoate was predominant in the microsomes while the NADH-linked reduction occurred mainly in the soluble fraction. Furthermore, phenobarbital or methylcholanthrene pretreatment stimulated the reductase activity only in the microsomes but not in the soluble fraction (147). The nitro reductase enzyme system was found to contain a flavoprotein as a prosthetic group, and addition of FMN (flavin mononucleotide), FAD (flavin adenenine dinucleotide) or riboflavin to the incubation mixture greatly enhanced the _in vitro_ metabolism (92, 148). Evidence has also been presented that NADPH-cytochrome c reductase is a component of the flavin-dependent nitro reductase (148, 149).

Gillette, Kamm and Sasame (150) have recently provided data to suggest that reduction of p-nitrobenzoate to p-aminobenzoate in liver microsomes is mediated by cytochrome P-450. This suggestion was based on evidence that: (a) carbon monoxide blocked nitro reduction, and the degree of inhibition was proportional to the amount of cytochrome P-450 bound as the carbon monoxide complex; and (b) the rate of nitro reduction was proportional to the amount of cytochrome P-450 in liver microsomes from animals previously treated with phenobarbital or carbon tetrachloride (150). Cytochrome P-450 was further implicated in the nitro reduction when it was shown that substances which bind with liver microsomes to cause a type II spectral change inhibited the biotransformation of p-nitrobenzoate by slowing the rate of cytochrome P-450

reduction (151).

Unlike most reductase enzymes, nitroreductase was found to be highly active under anaerobic conditions, but much less active in air (92, 148). This oxygen sensitivity has been ascribed, in part, to autoxidation of the hydroxylamine derivative formed as an intermediate in the reductive pathway (150). It has also been proposed that oxygen blocks nitroreductase activity by competing with the nitro substrate for the reduced form of cytochrome P-450 (150, 152).

III. EXPERIMENTAL METHODS AND MATERIALS

1. Animals

Adult male mice of the Swiss albino strain, 6 to 8 weeks of age and weighing 25 to 30 g were used. The animals were housed in plastic cages with bedding of spruce and poplar wood shavings and were allowed free access to Purina laboratory chow and water at all times. As closely as possible, lighting was regulated to allow 12 hours of uniform illumination and 12 hours of darkness each day. Upon arrival, the animals were kept in this quiet and relatively stress-free environment for at least three days before being divided by random selection into test and control groups. After the mice had been irradiated, their drinking water was acidified with hydrochloric acid to pH 2.8 to control any post-irradiation septicemia caused by *Pseudomonas aeruginosa* (153).

2. Chemicals and Reagents

All chemicals and reagents used in this investigation were of A.C.S. or analytical reagent grade quality.

Folin-Phenol Reagent:

1 ml of Folin-Phenol 2N solution (Fisher Scientific) diluted with 10 ml of distilled water.

Alkaline Copper Reagent:

1 ml 1% copper sulfate ($CuSO_4 \cdot 5H_2O$)
1 ml 2% potassium sodium tartrate
20 ml 10% sodium carbonate in 0.5 M NaOH

This reagent was always freshly prepared from stock solutions.

Nicotinamide adenine dinucleotide phosphate (NADP), glucose-

6-phosphate disodium and crystalline bovine serum albumin were purchased from Mann Research Laboratories, New York, U.S.A. Solutions of the various cofactors and substrates were prepared with distilled water and employed in the following concentrations: NADP - 4 μmoles per ml, glucose-6-phosphate - 50 μmoles per ml, $MgCl_2$ - 100 μmoles per ml, nicotinamide - 400 μmoles per ml, aniline - 20 μmoles per ml, aminopyrine - 10 μmoles per ml, and p-nitrobenzoic acid - 3 μmoles per ml. Semicarbazide HCl (100 μmoles per ml) was dissolved in 0.1 M phosphate buffer pH 7.4. The solutions of NADP, glucose-6-phosphate and aniline were always prepared on the same day in which they were used.

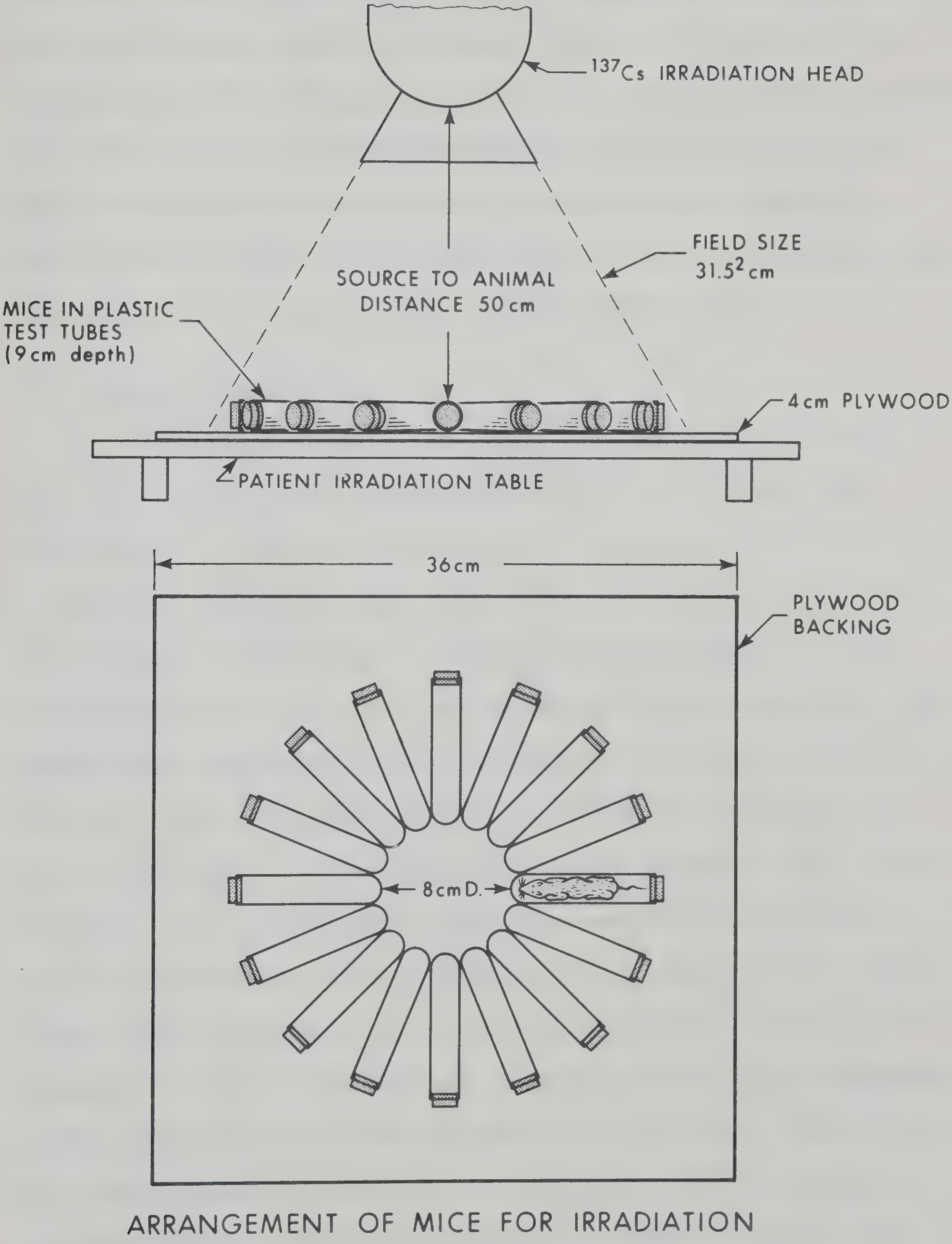
3. Irradiation Procedure

The mice were irradiated in groups of 16 with a theraputic 1,500 curie ^{137}Cs gamma source(a). The mice were placed individually in polyethylene test tubes provided with a large number of air holes and arranged on a 4 cm thickness of plywood as shown in Figure 2. The source-to-animal distance was 50 cm and gamma irradiation was delivered at 22.1R per minute. The radiation exposures were determined by placing a Victoreen condenser R-meter at various points in the polyethylene test tubes. Groups of mice were exposed to 300R, 600R, 950R and 1200R of whole-body gamma irradiation in a single dose. Sham-irradiated mice were used as controls.

(a) W.W. Cross Cancer Institute, Edmonton, Alberta.

Figure 2

Irradiation of Mice



4. Cold Stress

The mice were subjected to acute cold stress by placing them in individual plastic cages, with no bedding, and positioning them in the draft of a cold room fan for 9 hours at 2°C. These animals were closely watched towards the end of the cold exposure period and were removed as soon as they became prostrate. After removal from the cold room, the mice were again maintained in a stress-free environment for the duration of the experiment.

5. Tissue Preparation

After each mouse was sacrificed by decapitation, the gall bladder was removed and the entire liver was immediately excised and placed in a solution of ice-cold 1.15% KCl. The liver was then blotted, weighed and a 20% homogenate was prepared in cold 1.15% KCl using a Potter homogenizer fitted with a motor driven teflon pestle. The homogenate was centrifuged at 9,000 x g for 20 minutes at 4°C in a Servall refrigerated centrifuge to sediment cell debris, nuclei, mitochondria and any unbroken cells. The floating lipid layer was removed by aspiration and the 9,000 x g supernatant was transferred to clean glass test tubes and packed in ice. Aliquots of 3.5 ml of the 9,000 x g supernatant were transferred to polyethylene ultracentrifuge tubes, made up to a 10 ml volume with ice-cold 1.15% KCl, and centrifuged at 105,000 x g (average) for 60 minutes in a Beckman model L2-65B preparative ultracentrifuge (type 40 rotor) to sediment the microsomal fraction. The soluble

fraction (105,000 x g supernatant) was immediately decanted and 2 ml of ice-cold 0.1 M phosphate buffer pH 7.4 containing 10^{-3} M disodium EDTA was layered over the microsomal pellets. These tubes were kept in a refrigerator for determination of microsomal cytochrome P-450. To a second series of ultracentrifuge tubes were added 1 ml of the 9,000 x g supernatant and 9 ml of cold 1.15% KCl. After centrifugation for 60 minutes at 105,000 x g (average), the top layers were discarded, 2 ml of 1.15% KCl were added and the tubes set aside for determination of microsomal protein.

Microsomal protein and cytochrome P-450 were always determined within twelve hours after preparation of the microsomal pellets.

6. Enzyme Assays

The use of tissue homogenate methodology assumes that any chemical reaction which occurs in living cells will also occur in cell-free preparations of protoplasm provided that the conditions are right. It has been established that such an assumption is valid (154). For the accurate measurement of enzyme activity in cell fractions it is necessary that the activity observed in the *in vitro* incubations be a function of the concentration of that enzyme in the incubation mixture. In other words, enzyme activity should be measured under conditions in which minor variations in the concentration of substrates, coenzymes and electrolytes do not appreciably affect the results (154).

The enzyme assays used in the experimental portion of this thesis have all been adapted from several published methods. However, since these methods were devised for the study of certain enzyme systems in rat liver, it became necessary to alter some of the concentrations of liver homogenate and various cofactors when these procedures were applied to mouse liver. In preliminary studies, the amounts of liver fraction, as well as the other ingredients of the incubation mixtures, especially NADP and glucose-6-phosphate, were varied until it could be shown that the quantity of metabolite produced was primarily dependent on the amount of 9,000 x g liver supernatant added. In order to check whether the enzyme assays were sufficiently sensitive to detect changes in metabolic activity, groups of mice were injected intraperitoneally with sodium phenobarbital (50 mg/kg) to produce enzyme induction (83). The 2- to 3-fold increase that was observed in enzyme activity after three daily phenobarbital treatments indicated that these enzyme assay procedures were adequate. All enzyme assays were always begun within 30 minutes after preparation of the 9,000 x g supernatant.

Hepatic enzyme activity, microsomal protein, and cytochrome P-450 were determined at various periods after exposure to gamma radiation or cold. At each interval, 5 control and 7 test mice were sacrificed. The above-mentioned parameters were measured on day 1, 3, 5, 7, 11, 15 and 21 after 300R or 600R. Due to high mortality rates, the mice were not ordinarily maintained beyond the eleventh post-irradiation

day when doses of 950R or 1200R were administered. In the studies of drug-metabolizing systems following cold-stress, the same time intervals were used up to day 15, when recovery from the cold exposure appeared to be complete. Since a definite circadian rhythm in the activity of some hepatic drug-metabolizing enzymes has been demonstrated (155), the animals were generally sacrificed between 7:00 a.m. and 10:00 a.m. The limited size of mouse liver, as well as the lengthy enzyme assay procedures, made it necessary to divide the experimental work into two separate series in order to study the effects of irradiation and cold on: (a) oxidative pathways; and (b) a reductase system. In the first series, each liver of the control and test animals was assayed for the activity of aniline p-hydroxylase, aminopyrine N-demethylase, microsomal protein and microsomal cytochrome P-450. After completion of these experiments, a second series of control and test mice were used for the study of p-nitrobenzoic acid reductase, as well as microsomal protein and cytochrome P-450. Thus changes in microsomal protein and P-450 levels were noted under similar circumstances in two separate sets of experiments.

a. Determination of Aniline p-Hydroxylase

For the assay of aniline p-hydroxylase activity the following solutions were added to a 15 ml incubation flask: aniline 1.0 ml (20 μmoles), NADP 0.5 ml (2 μmoles), glucose-6-phosphate 0.5 ml (25 μmoles), $MgCl_2$ 0.25 ml (25 μmoles), nicotinamide 0.25 ml (100 μmoles), 9,000 x g supernatant 1.0 ml (equivalent to 200 mg of liver), and

0.1 M phosphate buffer pH 7.4 to make 5 ml. Incubation mixtures serving as blanks were also prepared containing all the components of the incubation mixture except the substrate. Incubation was carried out with shaking (70 oscillations per minute) in a Dubnoff apparatus for 30 minutes at 37°C under air. The assay procedure for measurement of the amount of p-aminophenol formed was essentially as described by Guarino *et al.* (57). The incubation reaction was stopped by adding the contents of the incubation flask to a 50 ml glass-stoppered centrifuge tube containing 1 g of sodium chloride and 20 ml of diethylether containing 1.5% isoamyl alcohol. The tubes were stoppered and mechanically shaken for 20 minutes to extract the metabolite into the organic phase. Fifteen-ml aliquots of the ether layer were transferred to another series of 50 ml centrifuge tubes which contained 1 ml of an aqueous solution of phenol 1.6% and 1 ml of 0.5 M Na_3PO_4. The tubes were stoppered, shaken for 15 minutes, and then another 8 ml of distilled water were added. The tubes were then shaken for 10 more minutes to allow for the development of the blue color by the indophenol reaction of the metabolite in the alkaline phenolic media. After centrifugation, the ether layer was aspirated and the optical density of the aqueous phase was measured at 620 mμ in a Beckman model DB spectrophotometer. The amount of p-aminophenol was calculated from a standard curve (Figure I, Appendix).

Preparation of a Standard Curve for p-Aminophenol:

Since p-aminophenol darkens rapidly upon exposure to light, the preparation of standard curves was done using solutions which were freshly prepared in the dark. When known amounts of p-aminophenol were added to 9,000 x g liver supernatant and extracted as described in the assay procedure, recoveries of 83% to 86% were obtained. The explanation was found in the work of Brodie and Axelrod (156), who showed that in a system containing 50 ml of ether and 5 ml of water about 85% of the p-aminophenol was distributed in the organic phase. The standard curve as shown in Figure I of the Appendix was obtained by adding known quantities of p-aminophenol to 1 ml of 9,000 x g liver supernatant and then carrying out the various steps of the assay procedure to determine the optical density at 620 mμ for various concentrations of p-aminophenol.

b. Determination of Aminopyrine N-Demethylase

The incubation mixture for the assay of hepatic aminopyrine N-demethylase activity consisted of: aminopyrine 1.0 ml (10 μmoles), NADP 0.25 ml (1 μmole), glucose-6-phosphate 0.25 ml (12 μmoles), $MgCl_2$ 0.25 ml (25 μmoles), nicotinamide 0.25 ml (100 μmoles), semicarbazide 0.5 ml (50 μmoles), 9,000 x g supernatant 0.5 ml (equivalent to 100 mg liver), and 0.1 M phosphate buffer pH 7.4 to make 6 ml. Blanks were prepared containing all of the above ingredients except aminopyrine. The flasks were incubated aerobically with shaking for 30 minutes at 37°C. The amount

of formaldehyde formed from the aminopyrine was determined by the method of Nash (157) as modified by Cochin and Axelrod (158). The incubation reaction was stopped by the addition of 2 ml of 20% zinc sulfate, followed after 5 minutes with 2 ml of saturated barium hydroxide solution. The contents of the incubation flasks were centrifuged and 5 ml aliquots of the protein-free supernatant were treated with 2 ml of double-strength Nash reagent (150 g ammonium acetate, 2 ml acetylacetone and distilled water to make 500 ml). After heating in a water bath at 60°C for 30 minutes to allow for color development, the solutions were cooled and the optical density at 415 mμ was read in a Beckman model DB spectrophotometer. The quantity of formaldehyde formed was calculated from a standard curve (Figure II, Appendix) which was prepared by adding known amounts of formaldehyde to incubation blanks and performing the assay procedure as outlined.

c. Determination of the p-Nitrobenzoic Acid Reductase

The assay of p-nitrobenzoic acid reductase activity has been shown to require a nitrogen atmosphere (92, 148). For this purpose, an extra incubation chamber was constructed of clear lucite and fitted into the water bath portion of the Dubnoff metabolic apparatus. A network of teflon tubing carried a stream of nitrogen which was directed onto the surface of the incubation mixture in each flask. Small holes on the top of the chamber allowed the excess nitrogen to escape and also permitted the addition of the

9,000 x g liver supernatant to the rest of the incubation media without loss of the nitrogen atmosphere.

To a series of 20 ml beakers were added: p-nitrobenzoic acid 2.0 ml (6 μmoles), NADP 0.5 ml (2 μmoles), glucose-6-phosphate 0.5 ml (25 μmoles), $MgCl_2$ 0.25 ml (25 μmoles), nicotinamide 0.25 ml (25 μmoles), and 0.1 M phosphate buffer pH 7.4 to make up a total of 5 ml. Incubation mixtures containing all the components except the substrate were used as blanks. The flasks containing these incubation mixtures were then placed in the lucite chamber and gassed with nitrogen for 15 minutes. Then the 9,000 x g supernatant (equivalent to 166.6 mg of liver) was added to each flask with a hypodermic syringe through the small holes on top of the special chamber. In this way, the nitrogen atmosphere was continually maintained. The mixtures were incubated under nitrogen for 30 minutes at 37°C with shaking in the specially adapted Dubnoff apparatus. The reaction was stopped by the addition of 4 ml of 20% trichloroacetic acid and the mixture was centrifuged for 10 minutes at about 3,000 x g. A 4 ml aliquot of the protein-free supernatant was used for measurement of the free p-aminobenzoic acid which was formed. Another 4 ml aliquot which was hydrolyzed by adding 0.3 ml of concentrated hydrochloric acid and heating on a boiling water bath for 30 minutes was used for the determination of the total (free plus conjugated) amount of metabolite. The amount of p-aminobenzoic acid was determined spectophotometrically according to the procedure

of Bratton and Marshall (159). To each 4 ml of the protein-free supernatant were added 1 ml of 0.1% sodium nitrite, 1 ml of 0.5% ammonium sulfamate, and 1 ml of 0.1% N-(1-napthyl)-ethylenediamine HCl solution at intervals of 10 minutes respectively. After 20 minutes, the optical density of the pink colored dye was measured at 540 mμ in a Beckman model DB spectrophotometer and the quantities of p-aminobenzoic acid were calculated from a standard curve (Figure III, Appendix). The standard curve was prepared by adding known concentrations of p-aminobenzoic acid to 1 ml of 9,000 x g supernatant and recovering the p-aminobenzoate according to the assay procedure.

7. Determination of Microsomal Protein

The measurement of protein in the microsomal pellets was based on the colorimetric method of Lowry *et al.* (160) as modified by Miller (161). The microsomal pellets were resuspended in distilled water by gentle homogenization with a small teflon pestle and brief shaking in a Vortex Jr. Mixer[a]. The resulting microsomal suspensions were quantitatively transferred from the ultracentrifuge tubes to volumetric flasks and diluted to 25 ml with distilled water. Duplicate 1 ml samples of the microsomal suspensions were pipetted into a series of test tubes for the determination of protein. After 1 ml of copper reagent was added to each

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[a] Scientific Industries Ltd., Queens Village, New York, U.S.A.

sample, the contents were well mixed and allowed to stand for 10 minutes at room temperature. Three milliliters of the diluted Folin-phenol solution were then added to each sample and the tubes were thoroughly mixed immediately upon addition of the reagent. The mixtures of protein and reagents were heated in a water bath for 10 minutes at 50°C and the resulting absorbance was read at 540 mμ in a Beckman model DB spectrophotometer. In order to determine whether the microsomal pellet was uniformly suspended in the 25 ml volume, serial dilutions of these suspensions were prepared and analyzed for protein content. A linear relationship was observed, indicating that the microsomal pellet was homogeneously distributed throughout the 25 ml volume. The quantity of microsomal protein was calculated from a standard curve prepared by using crystalline bovine serum albumin as the protein source (Figure IV, Appendix).

8. Determination of Cytochrome P-450

Microsomal cytochrome P-450 content was estimated essentially as described by Omura and Sato (37). The microsomal pellets were resuspended in 0.1 M PO_4 buffer pH 7.4 containing 10^{-3} M disodium EDTA, and diluted with the buffer to produce an approximate concentration of 2 to 3 mg protein per ml. This suspension was divided between two matched cuvettes fitted with ground-glass stoppers and a baseline was recorded using a Beckman model DB spectrophotometer by scanning from 500 to 400 mμ. Carbon monoxide was gently bubbled into the sample cuvette for 20 seconds,

and a few milligrams of crystalline sodium dithionite were added. The sample cuvette was then gassed with carbon monoxide for another minute. The reference cuvette was treated only with a few milligrams of sodium dithionite. The difference spectrum was then recorded from 500 mμ to 400 mμ and the cytochrome P-450 content was expressed as Δ O.D.$_{450-490\ \mathrm{m}\mu}$ per mg protein per milliliter.

9. Statistical Procedures

In the studies of the effects of irradiation and cold-stress on the activities of various drug-metabolizing enzyme systems, values for the mean, standard deviation and standard error of the mean were calculated by standard statistical procedures. Statistical comparisons between control and irradiated test groups were made by using the Student t-test (162). P values of <0.05 were considered to represent significant differences between means.

IV. RESULTS

1. Changes in Hepatic Oxidative Drug-Metabolizing Activity Following Whole-Body Exposures to Various Levels of Gamma Radiation

a. 300R

After 300R of gamma irradiation a cyclic response was observed in the activities of aniline p-hydroxylase and aminopyrine N-demethylase as well as in the microsomal protein and cytochrome P-450 levels (Tables 1, 2; Figure 3). No significant changes in any of these parameters were noted on the first post-irradiation day. However, on the third day, the activity of aniline p-hydroxylase increased to 42% above controls, aminopyrine N-demethylase rose to 32% and microsomal protein content was 18% higher than normal. The increase noted on the third day in the amount of microsomal cytochrome P-450 was not significant at the $P<0.05$ level. A decline slightly below control levels was seen in all four parameters on the fifth post-irradiation day. No further significant changes were observed in the microsomal protein content up to day 21. On day 7, cytochrome P-450, aniline p-hydroxylase and aminopyrine N-demethylase were 74%, 76% and 80% of controls respectively. The levels of cytochrome P-450 and aniline p-hydroxylase assumed normal values from day 11 until the end of the observation period. An over-compensatory increase to 118% of control values was observed in aminopyrine N-demethylase activity on day 11, with subsequent return to normal values on day 15 and 21.

Table 1

Effects of 300R on the *In Vitro* Metabolism of Aniline and Aminopyrine by Mouse Liver[a]

Post-Irradiation Time (Days)	Aniline p-hydroxylase[b]		Aminopyrine N-demethylase[c]	
	Control	Test	Control	Test
1	0.54 ± 0.03	0.59 ± 0.20	1.78 ± 0.11	1.82 ± 0.08
3	0.52 ± 0.02	0.73 ± 0.04[d]	1.65 ± 0.04	2.18 ± 0.11[d]
5	0.54 ± 0.04	0.50 ± 0.05	1.75 ± 0.09	1.64 ± 0.13
7	0.57 ± 0.01	0.44 ± 0.03[d]	1.85 ± 0.09	1.49 ± 0.09[d]
11	0.64 ± 0.01	0.65 ± 0.02	1.50 ± 0.09	1.79 ± 0.08[d]
15	0.51 ± 0.04	0.51 ± 0.03	1.40 ± 0.12	1.53 ± 0.07
21	0.54 ± 0.04	0.57 ± 0.03	1.69 ± 0.07	1.77 ± 0.10

a. Values in the table represent the mean ± standard error of the mean obtained from 5 control mice and 7 irradiated (test) mice at each post-irradiation period.

b. μmoles of aniline metabolized per gram of liver per 30 minutes.

c. μmoles of formaldehyde formed per gram of liver per 30 minutes.

d. Significantly different from controls at $P<0.05$ level.

Table 2

Effects of 300R on the Levels of Hepatic Microsomal Protein and Cytochrome P-450[a]

Post-Irradiation Time (Days)	Microsomal Protein[b]		Microsomal Cytochrome P-450[c]	
	Control	Test	Control	Test
1	10.67 ± 0.35	10.61 ± 0.58	0.066 ± 0.003	0.065 ± 0.002
3	10.95 ± 0.42	12.98 ± 0.61[d]	0.069 ± 0.002	0.079 ± 0.006
5	12.17 ± 0.56	11.23 ± 0.60	0.085 ± 0.001	0.078 ± 0.004
7	11.57 ± 0.40	10.71 ± 0.69	0.093 ± 0.004	0.070 ± 0.003[d]
11	12.18 ± 0.18	12.34 ± 0.31	0.088 ± 0.007	0.087 ± 0.002
15	10.95 ± 0.54	11.30 ± 0.49	0.097 ± 0.003	0.091 ± 0.005
21	11.50 ± 0.48	11.26 ± 0.50	0.088 ± 0.002	0.084 ± 0.002

a. Values in the table represent the mean ± standard error of the mean obtained from 5 control mice and 7 irradiated (test) mice at each post-irradiation interval.

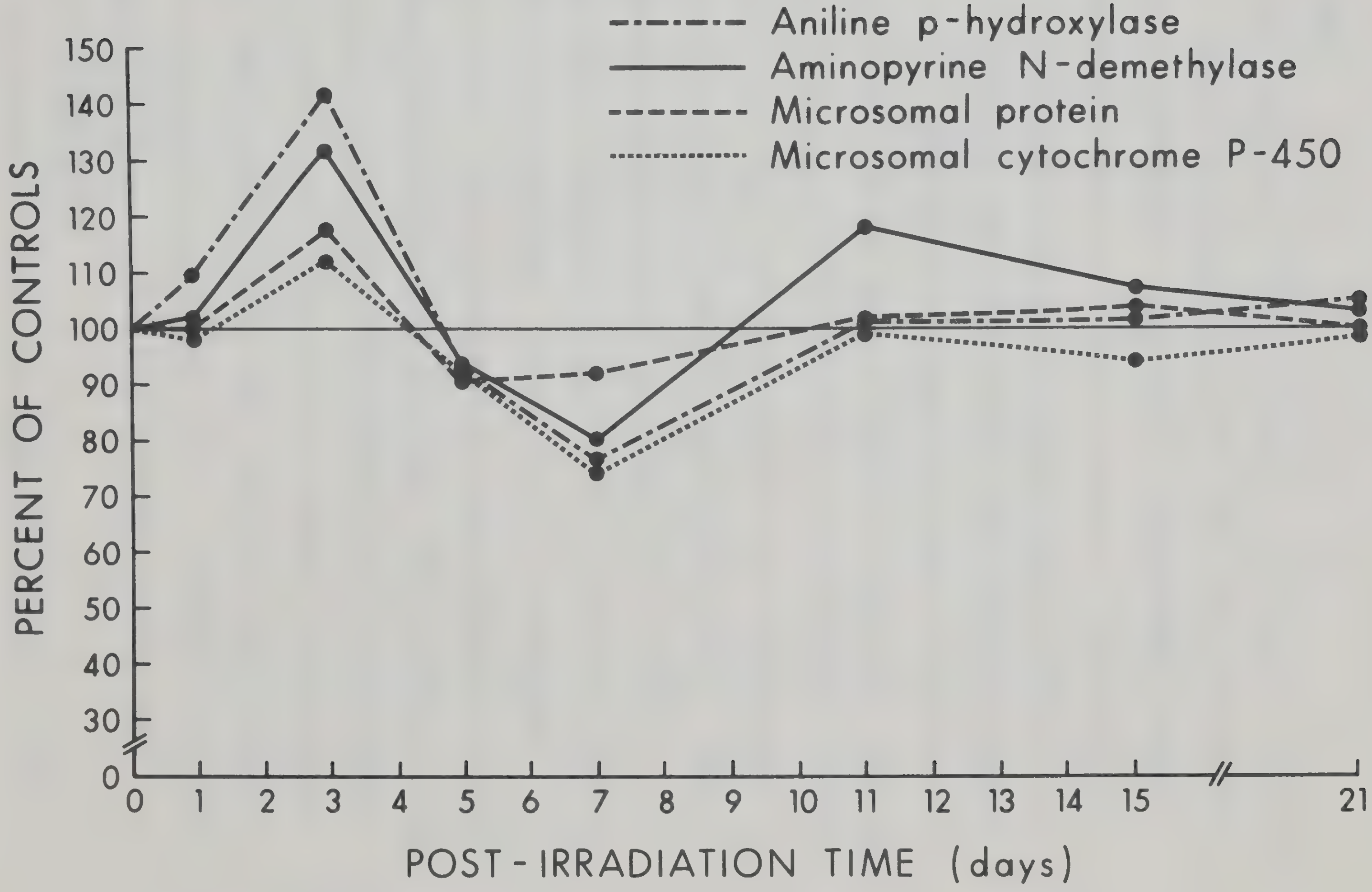
b. Milligrams of microsomal protein per gram of liver (wet weight).

c. Δ O.D. 450–490 mμ per mg protein per ml.

d. Statistically different from controls at $P<0.05$ level.

Figure 3

Effects of 300R Whole-Body Gamma Irradiation on Some
Hepatic Drug-Metabolizing Systems



No visible symptoms of radiation sickness were noted in any of the mice irradiated with 300R.

b. <u>600R</u>

The response to 600R (Tables 3, 4; Figure 4) followed a pattern similar to that seen with 300R, i.e. an initial stimulation followed by a decrease to sub-normal values and a subsequent return to control levels. The activity of aniline p-hydroxylase was 117% and 129% of controls on the first and third days after 600R, but declined abruptly to only 54% of normal by day 5 before gradually recovering to control levels by the end of the 21-day period. Similarly, the rate of aminopyrine N-demethylation which was elevated by 30% and 25% on the first and third days respectively also dropped to a low of 66% of controls on day 5. This was followed by a slow return to normal and a small overcompensatory increase on day 21. An increase of 10% was observed in microsomal protein on the first post-irradiation day, but the subsequent fluctuations were not significantly different from controls. The increases noted in microsomal cytochrome P-450 on day 1 and 3 after 600R were not statistically significant; however, P-450 levels were only 70% of controls on day 5 and fell continually, to 52% on the eleventh day. By day 21, however, the liver microsomes were found to contain a normal amount of cytochrome P-450 again.

In several of the mice treated with 600R, some gastro-intestinal damage was observed from the fifth day on,

Table 3

Effects of 600R on the *In Vitro* Metabolism of Aniline and Aminopyrine by Mouse Liver[a]

Post-Irradiation Time (Days)	Aniline p-hydroxylase[b]		Aminopyrine N-demethylase[c]	
	Control	Test	Control	Test
1	0.57 ± 0.02	0.67 ± 0.02[d]	1.03 ± 0.04	1.36 ± 0.06[d]
3	0.59 ± 0.02	0.77 ± 0.04[d]	1.16 ± 0.06	1.45 ± 0.07[d]
5	0.73 ± 0.07	0.40 ± 0.04[d]	2.06 ± 0.17	1.39 ± 0.15[d]
7	0.76 ± 0.03	0.50 ± 0.06[d]	2.30 ± 0.04	1.62 ± 0.20[d]
11	0.54 ± 0.03	0.40 ± 0.02[d]	1.53 ± 0.08	1.12 ± 0.05[d]
15	0.67 ± 0.05	0.62 ± 0.03	2.16 ± 0.17	2.04 ± 0.09
21	0.57 ± 0.06	0.61 ± 0.04	1.66 ± 0.05	1.90 ± 0.07[d]

a. Values in the table represent the mean ± standard error of the mean obtained from 5 control mice and 7 irradiated (test) mice at each post-irradiation period.

b. μmoles of aniline metabolized per gram of liver per 30 minutes.

c. μmoles of formaldehyde formed per gram of liver per 30 minutes.

d. Significantly different from controls at $P<0.05$ level.

Table 4

Effects of 600R on the Levels of Hepatic Microsomal Protein
and Cytochrome P-450[a]

Post-Irradiation Time (Days)	Microsomal Protein[b]		Microsomal Cytochrome P-450[c]	
	Control	Test	Control	Test
1	12.33 ± 0.25	12.95 ± 0.34	0.078 ± 0.001	0.085 ± 0.003
3	11.90 ± 0.46	13.09 ± 0.29[d]	0.077 ± 0.006	0.082 ± 0.002
5	11.38 ± 0.62	10.52 ± 0.33	0.092 ± 0.003	0.063 ± 0.005[d]
7	12.15 ± 0.41	11.30 ± 0.48	0.098 ± 0.004	0.062 ± 0.005[d]
11	10.70 ± 0.66	11.75 ± 0.33	0.105 ± 0.007	0.054 ± 0.004[d]
15	12.70 ± 0.85	13.28 ± 0.49	0.097 ± 0.005	0.088 ± 0.005
21	11.85 ± 0.85	13.22 ± 0.86	0.090 ± 0.003	0.089 ± 0.004

a. Values in the table represent the mean ± standard error of the mean obtained from 5 control mice and 7 irradiated (test) mice at each post-irradiation interval.

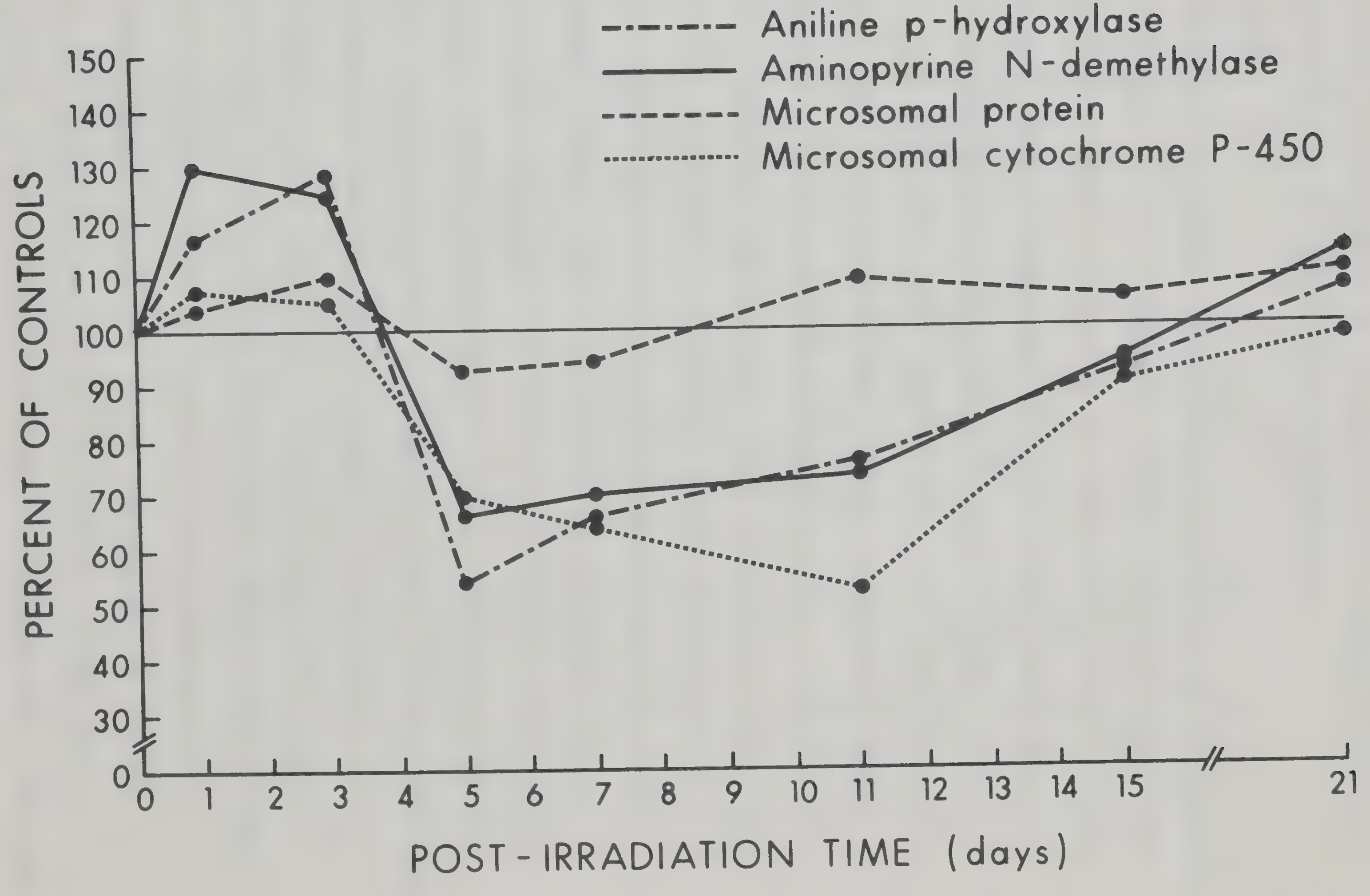
b. Milligrams of microsomal protein per gram of liver (wet weight).

c. Δ O.D. 450-490 mμ per mg protein per ml.

d. Statistically different from controls at $P<0.05$ level.

Figure 4

Effects of 600R Whole-Body Gamma Irradiation on Some Hepatic Drug-Metabolizing Systems



and a number of the irradiated animals died between the fifth and tenth day. However, the surviving mice appeared quite normal and healthy 21 days after irradiation.

c. <u>950R</u>

Twenty-four hours after 950R of whole-body irradiation, a sharp increase in the oxidative metabolism of aniline and aminopyrine was noted (Tables 5, 6; Figure 5). The aniline p-hydroxylase activity was 36% above controls while aminopyrine N-demethylase activity was elevated by 34%. Both enzyme levels remained above normal until the third post-irradiation day, but dropped very abruptly by day 5 when the metabolism of aniline and aminopyrine were only 74% and 50% of controls respectively. The activity of aniline p-hydroxylase declined continually to 41% of control at day 13, at which time aminopyrine N-demethylase was only 30% of normal values. Microsomal protein rose by 14% on day 1 and reached a peak of 20% above controls 3 days after 950R. On the fifth post-irradiation day, microsomal protein levels were essentially back to normal, but a further decrease to 75% of control was observed on day 7, followed by an apparent trend toward recovery. On the thirteenth post-irradiation day, there was no significant difference between microsomal protein content in control and test animals.

Microsomal cytochrome P-450 increased by 16% on the first day and then declined continually except for a small increase on the seventh day after irradiation. Thirteen days after 950R, the hepatic microsomal P-450 levels in the

Table 5

Effects of 950R on the *In Vitro* Metabolism of Aniline and Aminopyrine by Mouse Liver[a]

Post-Irradiation Time (Days)	Aniline p-hydroxylase[b]		Aminopyrine N-demethylase[c]	
	Control	Test	Control	Test
1	0.56 ± 0.04	0.77 ± 0.03[d]	1.18 ± 0.06	1.58 ± 0.09[d]
3	0.67 ± 0.04	0.86 ± 0.03[d]	1.00 ± 0.02	1.29 ± 0.09[d]
5	0.64 ± 0.04	0.47 ± 0.03[d]	1.47 ± 0.08	0.73 ± 0.20[d]
7	0.80 ± 0.05	0.52 ± 0.04[d]	1.78 ± 0.17	0.98 ± 0.09[d]
11	0.75 ± 0.07	0.36 ± 0.06[d]	2.38 ± 0.10	0.66 ± 0.13[d]
13	0.79 ± 0.05	0.32 ± 0.08[d]	2.03 ± 0.18	0.66 ± 0.19[d]

a. Values in the table represent the mean ± standard error of the mean obtained from 5 control mice and 7 irradiated (test) mice at each post-irradiation period.

b. μmoles of aniline metabolized per gram of liver per 30 minutes.

c. μmoles of formaldehyde formed per gram of liver per 30 minutes.

d. Significantly different from controls at $P<0.05$ level.

Table 6

Effects of 950R on the Levels of Hepatic Microsomal Protein and Cytochrome P-450[a]

Post-Irradiation Time (Days)	Microsomal Protein[b]		Microsomal Cytochrome P-450[c]	
	Control	Test	Control	Test
1	15.52 ± 0.40	17.69 ± 0.38^{d}	0.054 ± 0.003	0.063 ± 0.001^{d}
3	12.64 ± 0.66	15.29 ± 0.83^{d}	0.062 ± 0.005	0.063 ± 0.005
5	12.98 ± 0.70	13.61 ± 1.42	0.073 ± 0.005	0.047 ± 0.007^{d}
7	17.33 ± 0.79	12.64 ± 0.92^{d}	0.072 ± 0.006	0.052 ± 0.003^{d}
11	14.05 ± 0.73	11.64 ± 0.77^{d}	0.070 ± 0.008	0.032 ± 0.007^{d}
13	18.06 ± 0.88	16.21 ± 1.83	0.050 ± 0.003	0.016 ± 0.004^{d}

a. Values in the table represent the mean ± standard error of the mean obtained from 5 control mice and 7 irradiated (test) mice at each post-irradiation interval.

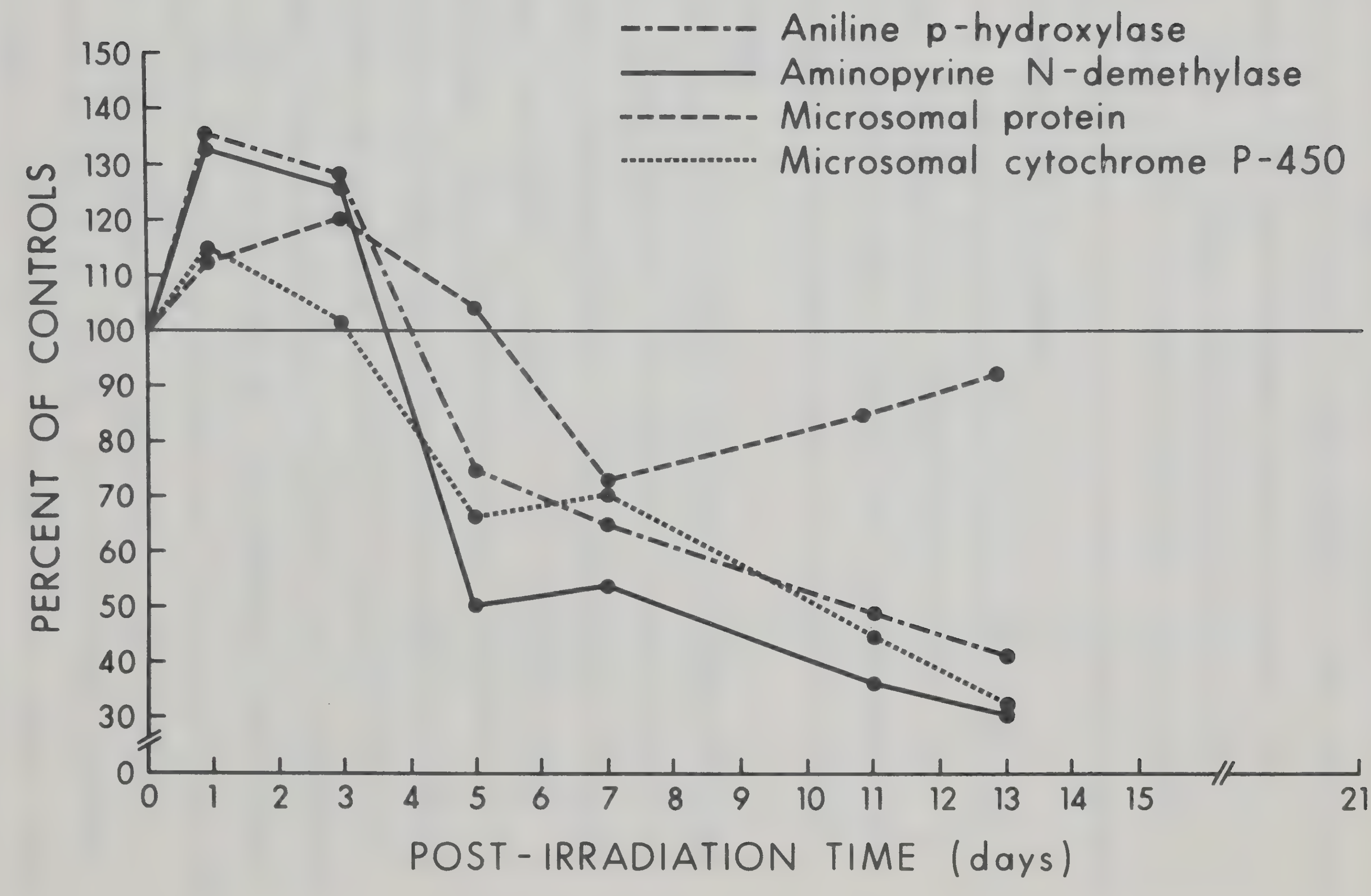
b. Milligrams of microsomal protein per gram of liver (wet weight).

c. Δ O.D. 450-490 mμ per mg protein per ml.

d. Statistically different from controls at $P<0.05$ level.

Figure 5

Effects of 950R Whole-Body Gamma Irradiation on Some
Hepatic Drug-Metabolizing Systems



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irradiated mice were only 32% of control values. It is noteworthy that although the level of cytochrome P-450 was normal on the third day, the metabolism of the substrates was elevated as was the microsomal protein content. In contrast, after the seventh post-irradiation day, the metabolic activity was observed to parallel the levels of cytochrome P-450, even though the microsomal protein appeared to be returning back to normal.

The irradiated mice were not maintained beyond day 13 due to high mortality rates. After the third day, the animals exhibited anorexia, diarrhea and listlessness, and upon dissection, gross gastrointestinal damage was evident.

d. <u>1200R</u>

The response of hepatic oxidative drug-metabolizing systems to 1200R (Tables 7, 8; Figure 6) differed from the changes seen after 950R mainly in that there was no initial stimulation, but rather a depression, on the first post-irradiation day. On day 1 following 1200R, the level of cytochrome P-450 dropped by 30% and the activity of aniline p-hydroxylase was only 82% of controls. The small drop in aminopyrine N-demethylation and the slight rise in microsomal protein on the first day were not statistically significant. Cytochrome P-450 levels after 1200R were continually depressed, reaching a low value of 40% of controls by the eleventh post-irradiation day. Microsomal protein content increased by 25% on day 3 and subsequently dropped to just below normal on day 5. It was interesting that the microsomal protein

Table 7

Effects of 1200R on the *In Vitro* Metabolism of Aniline and Aminopyrine by Mouse Liver[a]

Post-Irradiation Time (Days)	Aniline p-hydroxylase[b]		Aminopyrine N-demethylase[c]	
	Control	Test	Control	Test
1	0.64 ± 0.05	0.52 ± 0.03[d]	1.61 ± 0.10	1.48 ± 0.13
3	0.49 ± 0.03	0.57 ± 0.07	1.46 ± 0.13	1.65 ± 0.22
5	0.57 ± 0.04	0.42 ± 0.04[d]	1.75 ± 0.12	1.18 ± 0.07[d]
7	0.80 ± 0.07	0.54 ± 0.03[d]	2.29 ± 0.19	1.53 ± 0.07[d]
11	0.64 ± 0.03	0.26 ± 0.03[d]	2.22 ± 0.18	0.85 ± 0.05[d]

a. Values in the table represent the mean ± standard error of the mean obtained from 5 control mice and 7 irradiated (test) mice at each post-irradiation period.

b. μmoles of aniline metabolized per gram of liver per 30 minutes.

c. μmoles of formaldehyde formed per gram of liver per 30 minutes.

d. Significantly different from controls at $P<0.05$ level.

Table 8

Effects of 1200R on the Levels of Hepatic Microsomal Protein and Cytochrome P-450[a]

Post-Irradiation Time (Days)	Microsomal Protein[b]		Microsomal Cytochrome P-450[c]	
	Control	Test	Control	Test
1	14.50 ± 0.26	15.17 ± 0.80	0.045 ± 0.002	0.031 ± 0.004[d]
3	13.46 ± 0.66	17.12 ± 0.54[d]	0.063 ± 0.003	0.044 ± 0.003[d]
5	14.84 ± 0.67	13.48 ± 0.63	0.066 ± 0.002	0.041 ± 0.004[d]
7	14.80 ± 0.80	14.02 ± 0.30	0.065 ± 0.006	0.031 ± 0.005[d]
11	14.77 ± 0.69	13.66 ± 0.29	0.085 ± 0.006	0.035 ± 0.003[d]

a. Values in the table represent the mean ± standard error of the mean obtained from 5 control mice and 7 irradiated (test) mice at each post-irradiation interval.

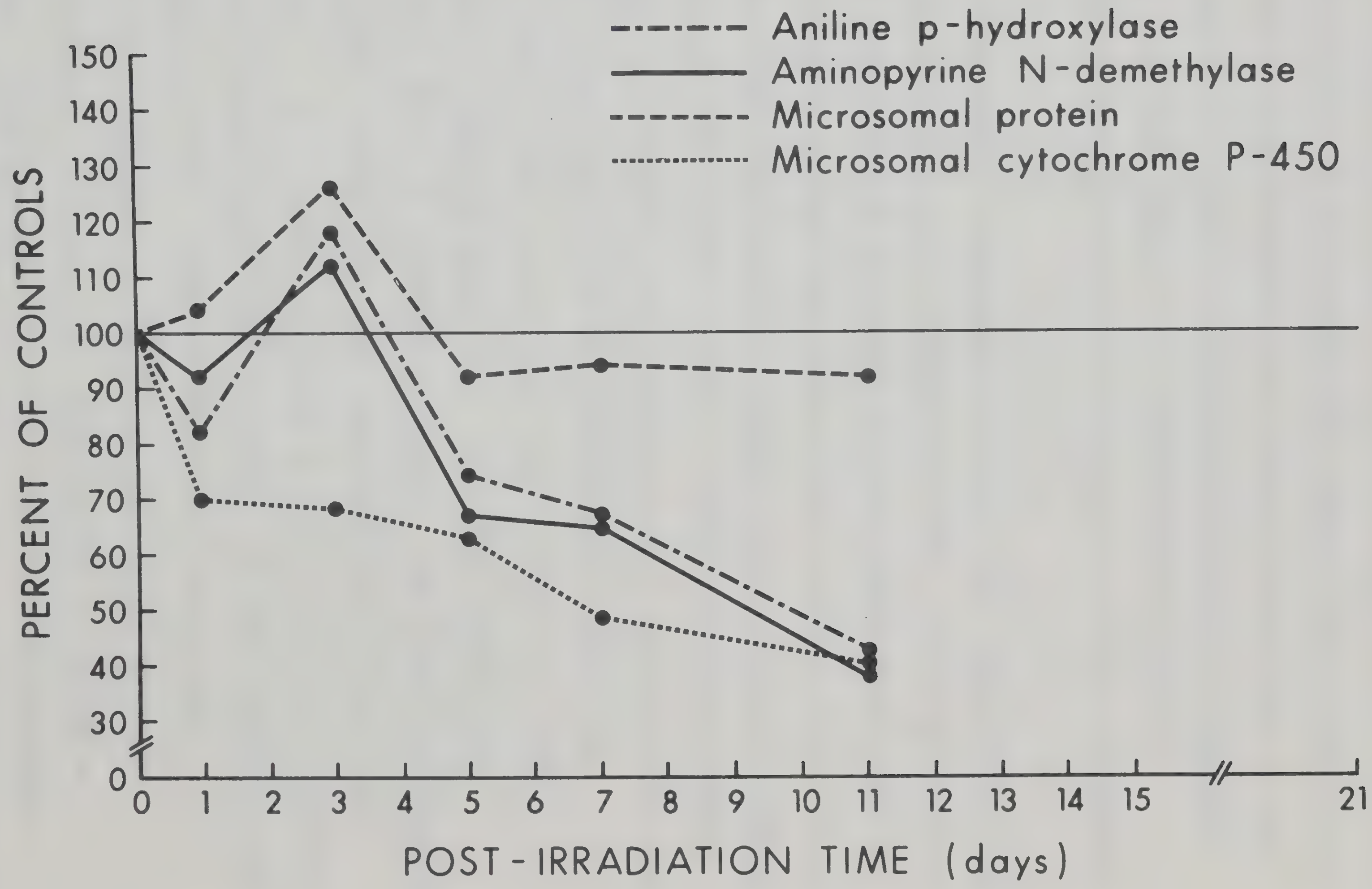
b. Milligrams of microsomal protein per gram of liver (wet weight).

c. Δ O.D. 450-490 mμ per mg protein per ml.

d. Statistically different from controls at $P<0.05$ level.

Figure 6

Effects of 1200R Whole-Body Gamma Irradiation on Some Hepatic Drug-Metabolizing Systems



content was not significantly changed on days 5, 7 and 11 even with this highly lethal radiation dosage. The increases observed in aniline and aminopyrine metabolism on day 3 were not statistically different from control values due to the large variations seen in the irradiated animals. Exposure to 1200R severely impaired the activities of aniline p-hydroxylase and aminopyrine N-demethylase after the third day.

The classic symptoms of lethal whole-body irradiation were obvious in the mice exposed to 1200R. After the second day, the animals were sluggish, ate and drank very little, developed diarrhea and lost appreciable weight. When the mice were sacrificed and dissected, gross gastrointestinal damage was visible. The largest number of deaths occurred between the fifth and seventh post-irradiation day, and the mice were not kept beyond day 11 due to the high mortality rate.

Some of the livers from mice treated with 950R or 1200R had an abnormal appearance after the third post-irradiation day. In several instances, the liver was blanched and the gall bladder was very large and full of greenish-black bile. In a number of other animals, the liver appeared very dark red and engorged with blood. In the determinations of cytochrome P-450, somewhat atypical spectra were occasionally observed in the liver microsomes from the irradiated mice. Figure V in the Appendix illustrates cytochrome P-450 spectra obtained from liver microsomes of mice exposed to 1200R as well as from control groups. It was

noted that the spectra of the carbon monoxide complex of dithionite-reduced microsomes from the control mice displayed a strong absorption band at 450 mμ with little or no absorption at 420 mμ. However, in the lethally irradiated mice, a number of spectra displayed prominent shoulders or secondary peaks at 420 mμ. This absorption at 420 mμ may have been due to contamination of the microsomal fraction with hemoglobin, since carboxyhemoglobin is known to have an absorption maximum at this wavelength (36). In spite of careful washing in ice-cold isotonic KCl solutions, all the blood could not be adequately removed from the livers of these irradiated mice, whereas such treatment effectively removed any hemoglobin contamination of the hepatic microsomes in the control animals.

2. Effects of Acute Cold-Stress on Hepatic Oxidative Drug Metabolism

Exposure of mice to acute cold-stress resulted in varying degrees of elevation in all four of the oxidative parameters with subsequent return to normal values (Tables 9, 10; Figure 7). Microsomal protein levels were 25% above controls twenty-four hours after removing the mice from the cold room. The protein content of the liver microsomes was essentially back to normal by the third day and did not deviate significantly from control levels for the remainder of the 15-day observation period. Microsomal cytochrome P-450 was elevated somewhat more slowly than protein levels, the increase in P-450 on day 1 not being

Table 9

Effects of Cold Stress on the *In Vitro* Metabolism of Aniline and Aminopyrine by Mouse Liver[a]

Post-Stress Time (Days)	Aniline p-hydroxylase[b]		Aminopyrine N-demethylase[c]	
	Control	Test	Control	Test
1	0.41 ± 0.02	0.64 ± 0.06[d]	1.22 ± 0.08	1.64 ± 0.11[d]
3	0.49 ± 0.03	0.49 ± 0.05	1.58 ± 0.03	1.91 ± 0.15[d]
5	0.46 ± 0.02	0.57 ± 0.04[d]	1.60 ± 0.07	1.61 ± 0.12
7	0.65 ± 0.02	0.63 ± 0.04	1.52 ± 0.04	1.56 ± 0.07
11	0.59 ± 0.02	0.69 ± 0.02[d]	1.89 ± 0.14	2.11 ± 0.07
15	0.48 ± 0.03	0.46 ± 0.02	1.48 ± 0.06	1.49 ± 0.06

a. Values in the table represent the mean ± standard error of the mean obtained from 5 control mice and 7 stressed (test) mice at each post-exposure period.

b. μmoles of aniline metabolized per gram of liver per 30 minutes.

c. μmoles of formaldehyde formed per gram of liver per 30 minutes.

d. Significantly different from controls at $P<0.05$ level.

Table 10

Effects of Cold Stress on the Levels of Hepatic Microsomal Protein and Cytochrome P-450[a]

Post-Stress Time (Days)	Microsomal Protein[b]		Microsomal Cytochrome P-450[c]	
	Control	Test	Control	Test
1	13.01 ± 0.72	16.31 ± 0.97[d]	0.044 ± 0.006	0.058 ± 0.008
3	13.50 ± 0.15	14.10 ± 0.78	0.052 ± 0.002	0.068 ± 0.002[d]
5	13.25 ± 0.74	12.71 ± 0.46	0.053 ± 0.003	0.068 ± 0.002[d]
7	12.10 ± 0.45	12.16 ± 0.56	0.067 ± 0.005	0.062 ± 0.002
11	15.37 ± 0.69	14.20 ± 0.81	0.059 ± 0.005	0.072 ± 0.004
15	11.20 ± 0.39	10.96 ± 0.36	0.068 ± 0.002	0.064 ± 0.003

a. Values in the table represent the mean ± standard error of the mean obtained from 5 control mice and 7 stressed (test) mice at each post-exposure interval.

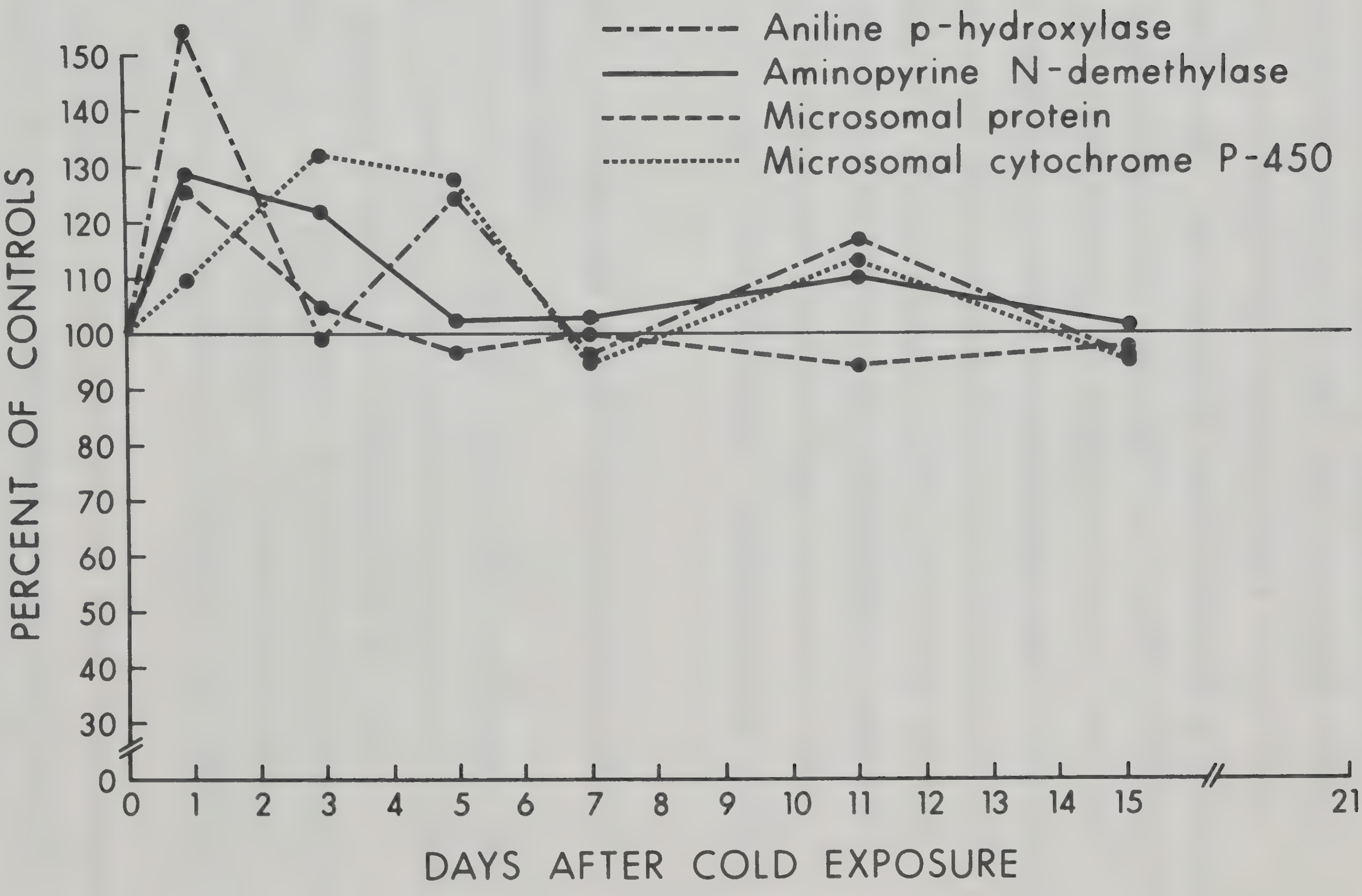
b. Milligrams of microsomal protein per gram of liver (wet weight).

c. Δ O.D.$_{450\text{-}490\ m\mu}$ per mg protein per ml.

d. Statistically different from controls at $P<0.05$ level.

Figure 7

Effects of Acute Cold-Stress on Some Hepatic Drug-Metabolizing Systems



statistically significant at the $P<0.05$ level. However, on day 3 and day 5, the levels of the hemeprotein were 32% and 28% above control values. The cytochrome P-450 content was not appreciably changed on days 7, 11 and 15 after cold-stress. The levels of hepatic aniline p-hydroxylase in the stressed mice were portrayed by a series of increases interspersed with intervals of normal activity. Thus the _in vitro_ metabolism of aniline was elevated 54% on the first day, 24% on the fifth day and 16% on the eleventh day, with normal activity being observed in the intervening periods. The metabolism of aminopyrine was significantly enhanced on the first and third days, and then returned to normal except for a small overcompensatory increase on the eleventh day.

3. Effects of Whole-Body Irradiation on a Hepatic Nitroreductase System

The ability of ionizing radiation to influence the activity of hepatic nitroreductase was of particular interest because previous investigators had reported that the development of reductase enzymes in the livers of growing rats was not affected by x-irradiation in doses which severely impaired the normal development of oxidative drug-metabolizing enzymes (120, 121, 122).

Since hepatic nitro reduction was shown to be mediated by cytochrome P-450 (150), each assay for the estimation of p-nitrobenzoic acid reductase was also accompanied by measurements of microsomal protein and cytochrome P-450

in the same liver sample to ascertain if alterations in enzyme activity could be correlated with changes in the content of protein or cytochrome P-450. Thus, in this investigation, protein and cytochrome P-450 in liver microsomes were determined in two separate series of experiments: those studying the oxidative pathways, and those involved with the reductive route of drug metabolism. Such a duplication in measurements of protein and cytochrome P-450 under similar circumstances in two distinct sets of experiments provided additional confirmation of the results. The responses of microsomal protein and P-450 levels to radiation and cold as seen in the series dealing with p-nitrobenzoic acid reductase (Figures 8 to 12) essentially followed the same patterns as described in the study of the oxidative drug-metabolizing enzymes (Figures 3 to 7).

a. 300R

After 300R, the *in vitro* metabolism of p-nitrobenzoic acid was significantly changed only on the third post-irradiation day, when the activity was 11% above control values (Table 11; Figure 8). The small decline in nitro reduction on day 5 was not statistically significant at the $P<0.05$ level and the enzyme activity did not deviate appreciably from normal values throughout the remainder of the 15-day observation period.

b. 600R

The activity of p-nitrobenzoic acid reductase increased by 16% and 36% on the first and third days after 600R, followed

Table 11

Effects of 300R Whole-Body Gamma Irradiation on a Hepatic Nitroreductase System[a]

Time[b]	p-Nitrobenzoic Acid Reductase[c]		Microsomal Protein[d]		Microsomal Cytochrome P-450[e]	
	Control	Test	Control	Test	Control	Test
1	0.93 ± 0.03	0.91 ± 0.06	14.95 ± 0.45	14.88 ± 0.60	0.074 ± 0.005	0.073 ± 0.002
3	1.00 ± 0.06	1.14 ± 0.02[f]	14.99 ± 0.49	16.44 ± 0.37[f]	0.068 ± 0.004	0.079 ± 0.002[f]
5	0.95 ± 0.05	0.87 ± 0.02	12.80 ± 0.40	14.12 ± 0.73	0.083 ± 0.003	0.082 ± 0.004
7	1.13 ± 0.06	0.98 ± 0.06	16.64 ± 0.91	16.79 ± 0.69	0.076 ± 0.004	0.063 ± 0.004[f]
11	0.91 ± 0.04	0.85 ± 0.02	14.57 ± 0.54	14.47 ± 0.35	0.078 ± 0.002	0.073 ± 0.003
15	0.97 ± 0.05	1.02 ± 0.06	13.42 ± 0.47	14.76 ± 0.36	0.080 ± 0.003	0.080 ± 0.002

a. Values in the table represent the mean ± standard error of the mean obtained from 5 control mice and 7 irradiated (test) mice.

b. Days after irradiation

c. μmoles of p-aminobenzoic acid (free and conjugated) formed per gram of liver per 30 minutes.

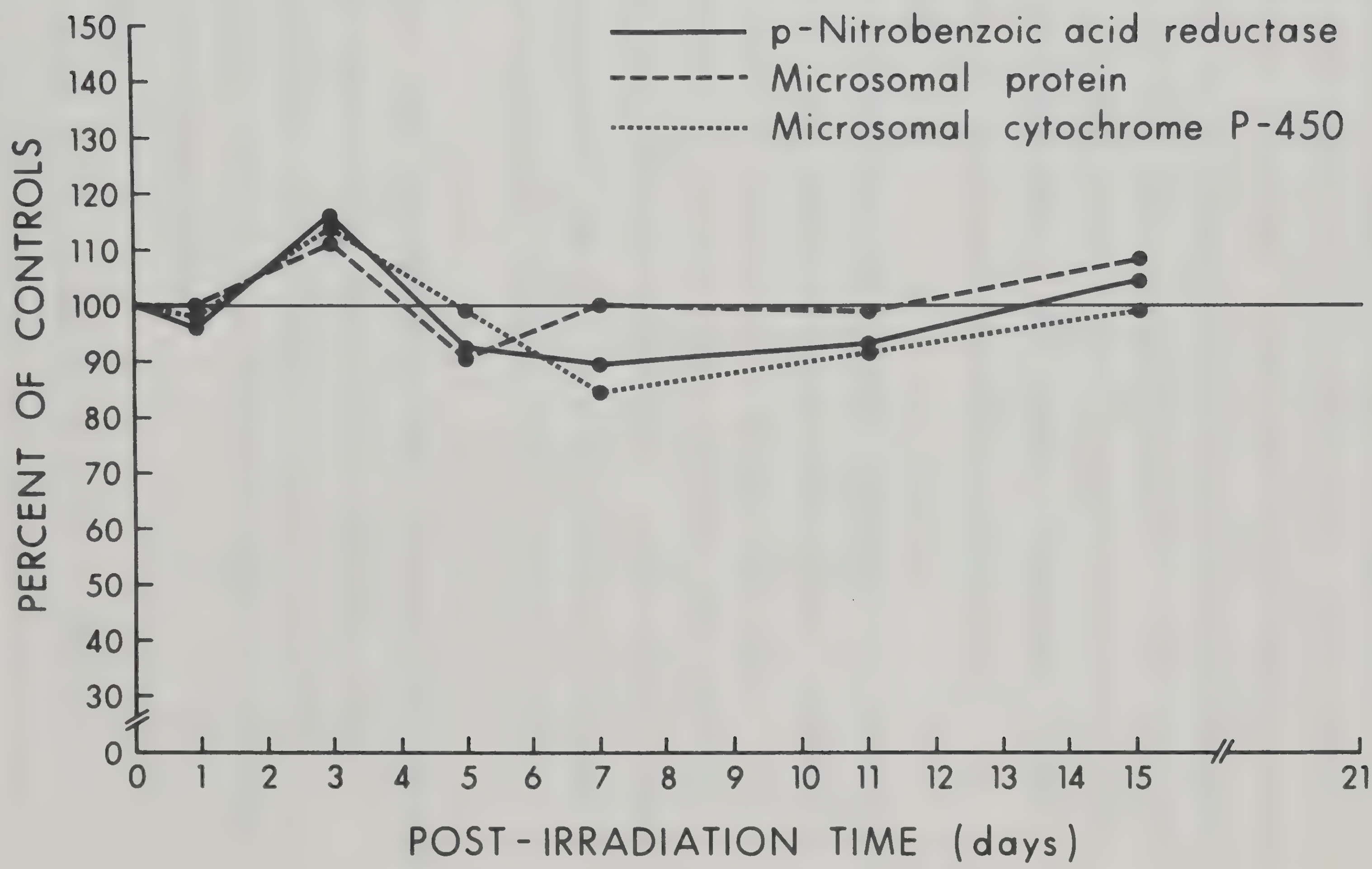
d. Milligrams of microsomal protein per gram of liver (wet weight).

e. Δ O.D. 450-490 mμ per mg protein per ml.

f. Statistically different from controls at $P<0.05$ level.

Figure 8

Effects of 300R Whole-Body Gamma Irradiation on a Hepatic Nitroreductase System



by a gradual decline to a low of 65% of controls on the eleventh day, with eventual recovery to normal values by the twenty-first day (Table 12; Figure 9). In this series of experiments, microsomal protein was significantly elevated on the first and third post-irradiation days with subsequent return to control levels. As also observed in the first set of data, cytochrome P-450 content increased slightly on the first and third day after 600R, dropped considerably from day 3 to day 11, but assumed normal values by day 21.

c. 950R

An initial stimulatory phase in hepatic p-nitrobenzoic acid metabolism was seen twenty-four hours after irradiation with 950R (Table 13; Figure 10). By the third post-irradiation day this increase was no longer statistically significant and a further abrupt fall to below 50% of normal activity was noted on day 5. This low level of reductase activity was maintained throughout the remainder of the experiment.

After 950R, microsomal protein increased and subsided in a cyclic pattern, but normal levels of protein were maintained in the liver microsomes by the eleventh day. A marked depletion in hepatic cytochrome P-450 was noted after the first day following 950R.

d. 1200R

In spite of the immediate depression observed in microsomal cytochrome P-450 due to irradiation with 1200R, the activity of p-nitrobenzoic acid reductase increased

Table 12

Effects of 600R Whole-Body Gamma Irradiation on a Hepatic Nitroreductase System[a]

Time[b]	p-Nitrobenzoic Acid Reductase[c]		Microsomal Protein[d]		Microsomal Cytochrome P-450[e]	
	Control	Test	Control	Test	Control	Test
1	1.08 ± 0.03	1.22 ± 0.04[f]	13.67 ± 0.38	15.26 ± 0.37[f]	0.073 ± 0.002	0.081 ± 0.001[f]
3	0.86 ± 0.03	1.17 ± 0.05[f]	13.55 ± 0.15	16.98 ± 0.47[f]	0.073 ± 0.002	0.082 ± 0.005
5	1.15 ± 0.07	1.02 ± 0.09	17.37 ± 0.78	18.42 ± 0.80	0.074 ± 0.002	0.064 ± 0.004
7	1.08 ± 0.04	0.78 ± 0.07[f]	16.07 ± 0.68	15.37 ± 0.50	0.077 ± 0.002	0.057 ± 0.006[f]
11	1.09 ± 0.05	0.73 ± 0.08[f]	13.82 ± 0.60	14.30 ± 0.57	0.082 ± 0.003	0.057 ± 0.006[f]
15	1.00 ± 0.04	0.86 ± 0.01[f]	14.17 ± 0.60	14.55 ± 0.32	0.081 ± 0.002	0.069 ± 0.002[f]
21	0.96 ± 0.03	0.91 ± 0.05	14.05 ± 0.42	14.42 ± 0.40	0.082 ± 0.002	0.076 ± 0.002

a. Values in the table represent the mean ± standard error of the mean obtained from 5 control mice and 7 irradiated (test) mice.

b. Days after irradiation.

c. µmoles of p-aminobenzoic acid (free and conjugated) formed per gram of liver per 30 minutes.

d. Milligrams of microsomal protein per gram of liver (wet weight).

e. Δ O.D. 450-490 mµ per mg protein per ml.

f. Statistically different from controls at $P<0.05$ level.

Figure 9

Effects of 600R Whole-Body Gamma Irradiation on a Hepatic Nitroreductase System

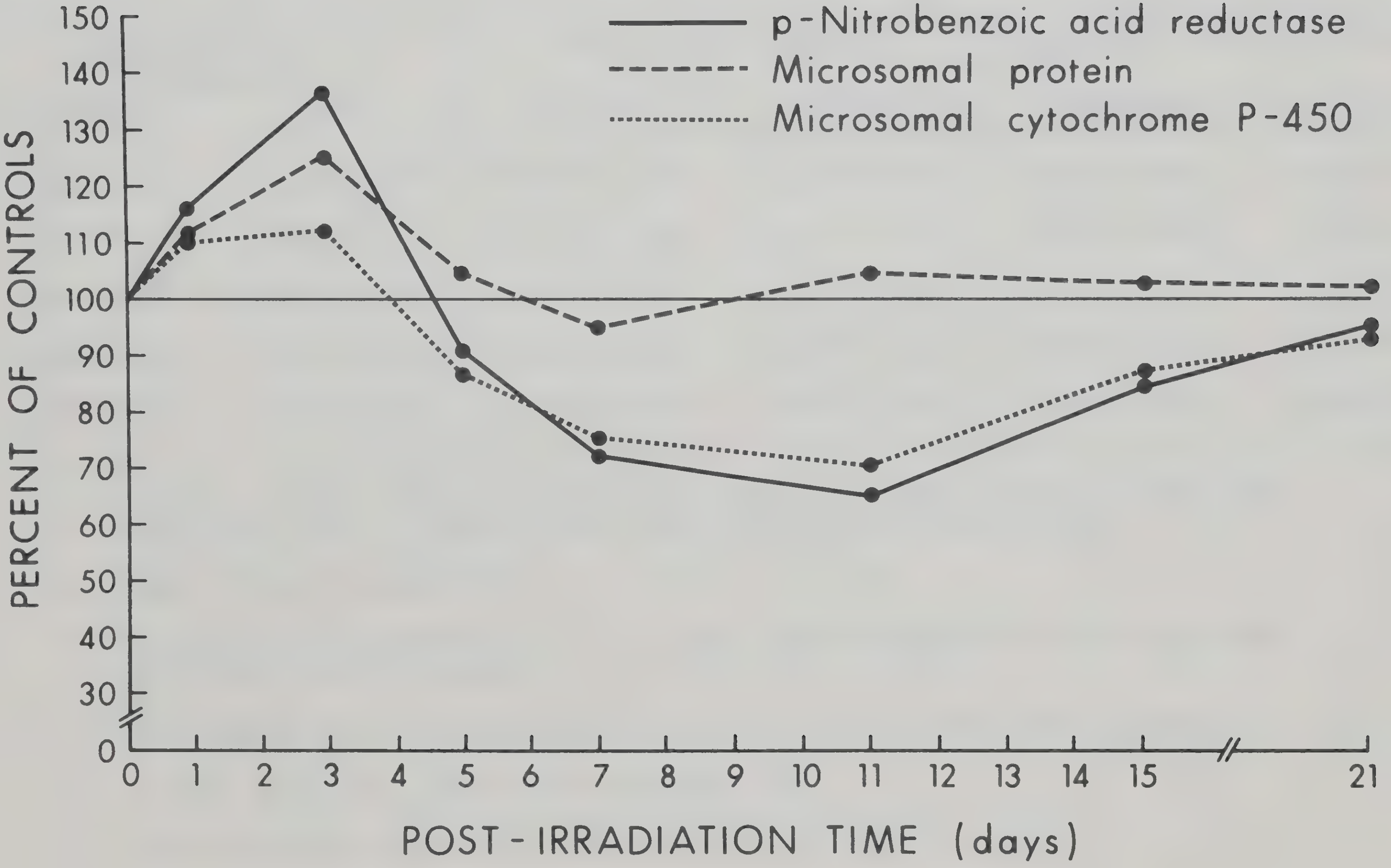


Table 13

Effects of 950R Whole-Body Gamma Irradiation on a Hepatic Nitroreductase System[a]

Time[b]	p-Nitrobenzoic Acid Reductase[c]		Microsomal Protein[d]		Microsomal Cytochrome P-450[e]	
	Control	Test	Control	Test	Control	Test
1	1.02 ± 0.04	1.20 ± 0.04[f]	14.47 ± 0.62	16.17 ± 0.23[f]	0.082 ± 0.001	0.086 ± 0.002
3	0.96 ± 0.01	1.00 ± 0.09	14.77 ± 0.36	18.11 ± 0.78[f]	0.088 ± 0.005	0.067 ± 0.004[f]
5	1.10 ± 0.05	0.53 ± 0.06[f]	14.30 ± 0.64	12.68 ± 0.78	0.089 ± 0.003	0.062 ± 0.004[f]
7	1.04 ± 0.11	0.49 ± 0.05[f]	13.47 ± 0.35	11.68 ± 0.32	0.089 ± 0.005	0.058 ± 0.006[f]
11	1.07 ± 0.05	0.52 ± 0.03[f]	17.30 ± 0.98	16.62 ± 0.44	0.083 ± 0.005	0.040 ± 0.007[f]

a. Values in the table represent the mean ± standard error of the mean obtained from 5 control mice and 7 irradiated (test) mice.

b. Days after irradiation.

c. μmoles of p-aminobenzoic acid (free and conjugated) formed per gram of liver per 30 minutes.

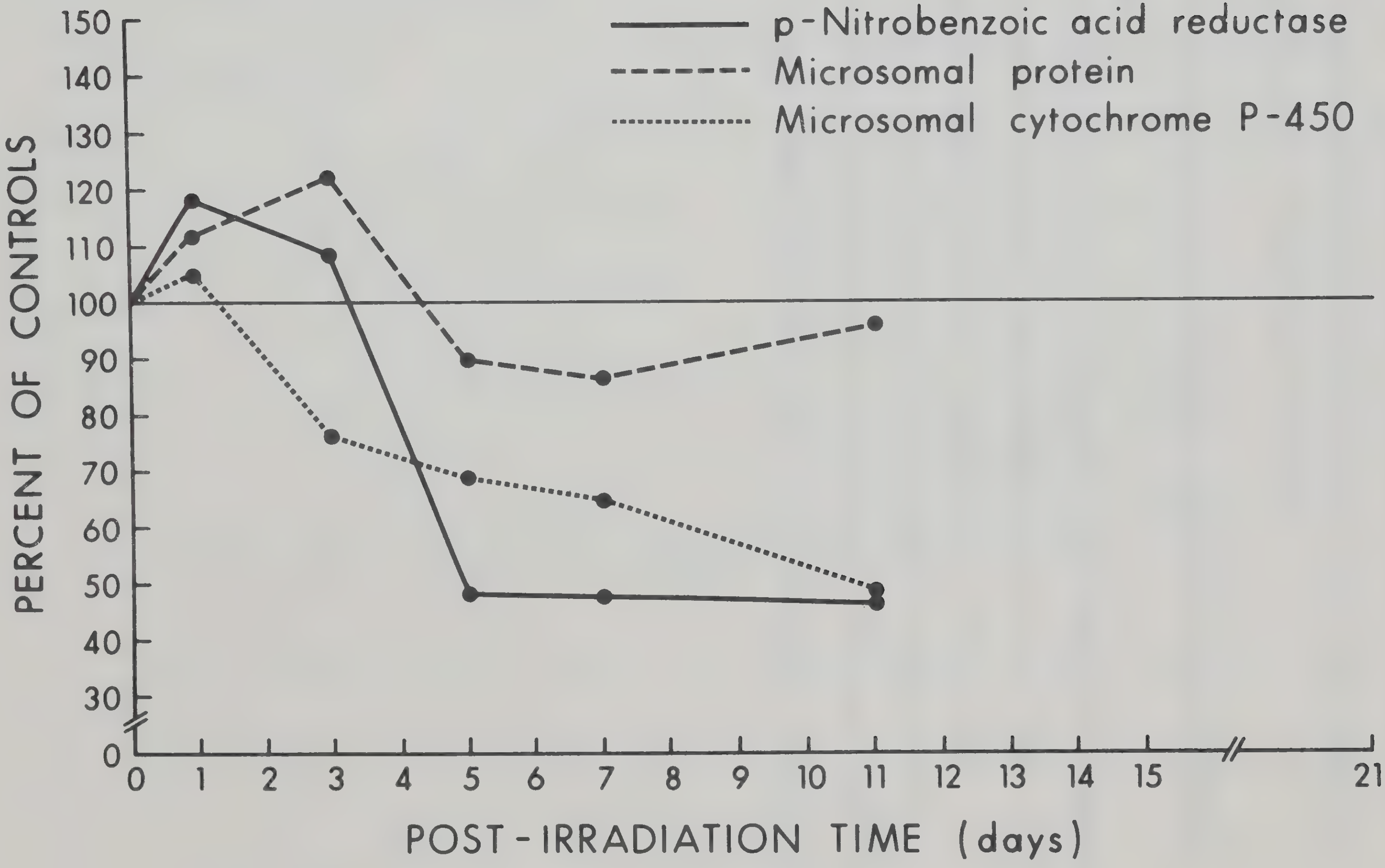
d. Milligrams of microsomal protein per gram of liver (wet weight).

e. Δ O.D. 450-490 mμ per mg protein per ml.

f. Statistically different from controls at $P<0.05$ level.

Figure 10

Effects of 950R Whole-Body Gamma Irradiation on a Hepatic Nitroreductase System



on day 1 and 3, as did the microsomal protein content (Table 14; Figure 11). However, after the third post-irradiation day, the severe reduction in P-450 levels was paralleled by a sharp impairment in reductase activity.

4. Effects of Acute Cold-Stress on a Hepatic Nitroreductase System

Exposure to cold significantly enhanced the metabolism of p-nitrobenzoic acid by liver homogenate up to 5 days after the stress condition (Table 15; Figure 12). Microsomal protein content was increased on the first and third day, while the levels of microsomal cytochrome P-450 were statistically elevated on the third and fifth day following the cold stress.

Table 14

Effects of 1200R Whole-Body Gamma Irradiation on a Hepatic Nitroreductase System[a]

Time[b]	p-Nitrobenzoic Acid Reductase[c]		Microsomal Protein[d]		Microsomal Cytochrome P-450[e]	
	Control	Test	Control	Test	Control	Test
1	0.91 ± 0.04	0.98 ± 0.02	14.97 ± 0.65	17.44 ± 0.49[f]	0.108 ± 0.003	0.087 ± 0.004[f]
3	0.83 ± 0.02	0.99 ± 0.02[f]	13.62 ± 0.36	17.26 ± 0.41[f]	0.105 ± 0.004	0.088 ± 0.003[f]
5	1.17 ± 0.06	0.57 ± 0.06[f]	14.24 ± 0.77	12.46 ± 0.85	0.107 ± 0.003	0.053 ± 0.005[f]
7	1.12 ± 0.05	0.60 ± 0.02[f]	14.01 ± 0.33	12.05 ± 0.59[f]	0.091 ± 0.003	0.056 ± 0.004[f]
10	1.10 ± 0.04	0.67 ± 0.03[f]	13.85 ± 0.43	12.75 ± 0.51	0.097 ± 0.006	0.053 ± 0.003[f]

a. Values in the table represent the mean ± standard error of the mean obtained from 5 control mice and 7 irradiated (test) mice.

b. Days after irradiation.

c. μmoles of p-aminobenzoic acid (free and conjugated) formed per gram of liver per 30 minutes.

d. Milligrams of microsomal protein per gram of liver (wet weight).

e. Δ O.D. 450-490 mμ per mg protein per ml.

f. Statistically different from controls at $P<0.05$ level.

Figure 11

Effects of 1200R Whole-Body Gamma Irradiation on a Hepatic Nitroreductase System

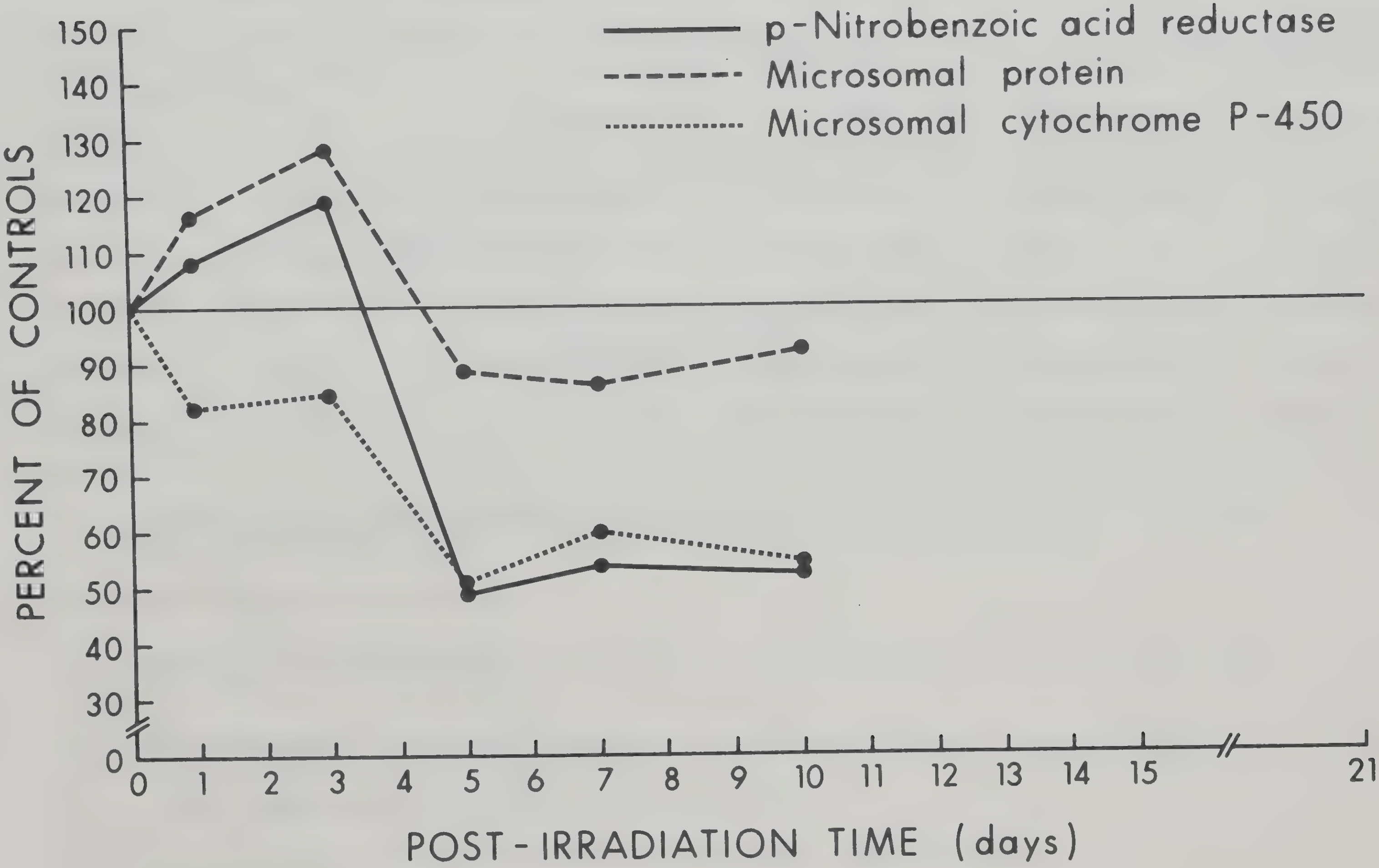
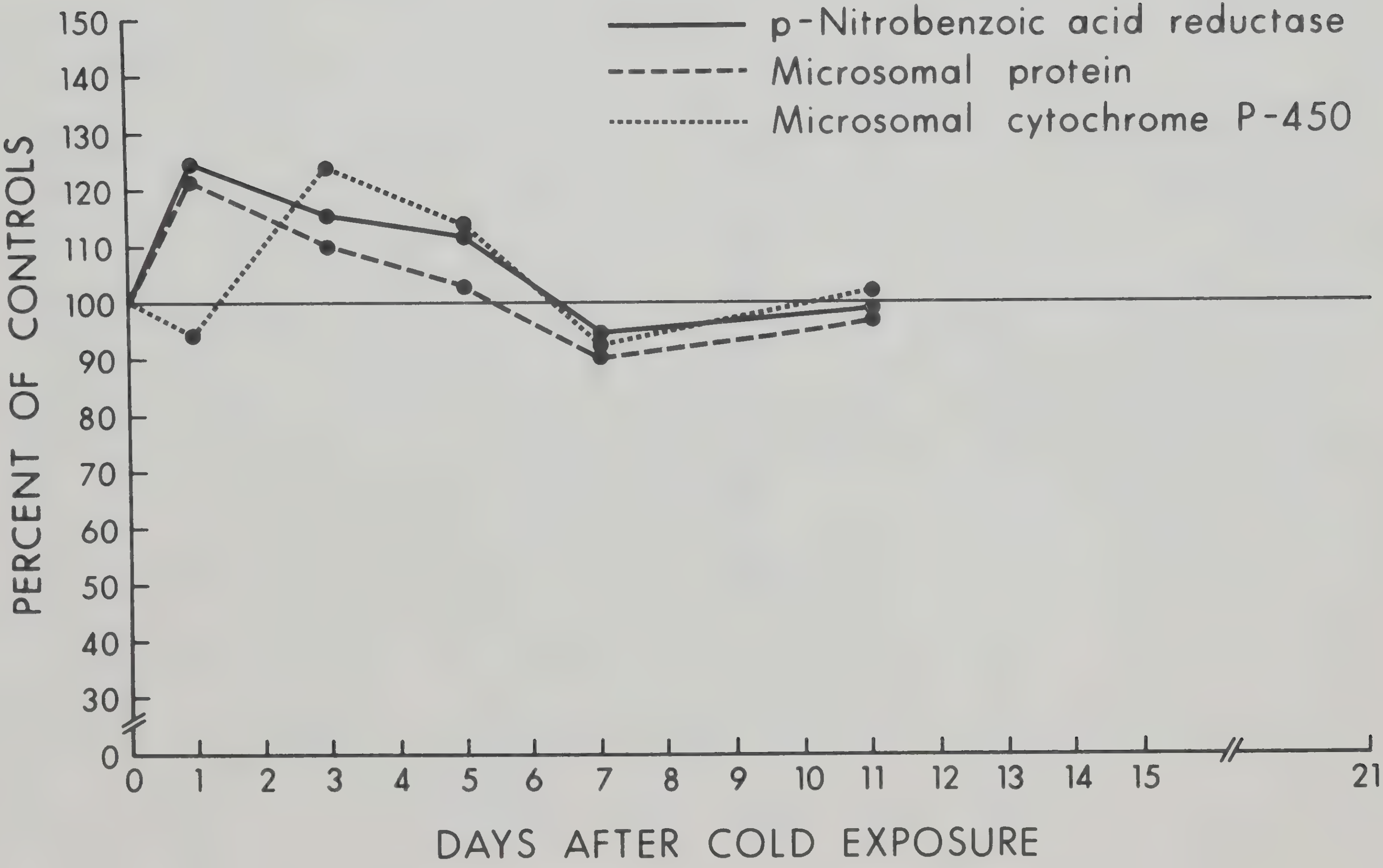


Figure 12

Effects of Acute Cold-Stress on a Hepatic Nitroreductase System



It is evident from the data presented in the previous section that two crucial points in the evaluation of the radiosensitivity of hepatic drug-metabolizing enzymes are the time-dependence and the dose-dependence of the phenomenon. The effect of whole-body irradiation on hepatic drug metabolism cannot be determined adequately merely by studying at one or two points after irradiation. The present results indicate that if measurements are performed at times before or after the maximal effects occur, one might conclude that the drug-metabolizing enzymes in the liver are not sensitive to irradiation. For example, the *in vitro* metabolism of aniline and aminopyrine or p-nitrobenzoic acid may have appeared normal if measured only on day 4 after 950R (Figures 5 and 10) or 1200R (Figures 6 and 11). The dose-dependence of the hepatic detoxication enzymes was illustrated by the recovery of these systems from sublethal doses of irradiation, and by continued impairment following exposure to high levels of gamma radiation.

It is possible that several investigators who have reported that irradiation did not affect hepatic drug-metabolizing capacity may have based their views on measurements that were taken at a point when changes were not obvious. Hietbrink and DuBois (120, 123) stated that radiation did not alter the ability of adult animals to detoxify foreign chemicals. However, it must be noted that these conclusions were reached by performing *in vitro* assays on the eighth,

thirteenth, twenty-second and twenty-seventh day after exposure of adult rats to 400R of x-irradiation. The results presented in Figure 3 and 8 suggest that the hepatic systems in the rats may have already recovered sufficiently by the eighth to twenty-seventh post-irradiation day so that no significant changes could be observed. It is quite possible that an effect would have been noted prior to day eight. Terayama and Takata (128) reported that neither 440R or 880R impaired the aminoazo N-demethylase system in rat liver, yet these observations were based on the assay of enzyme activity only at 35 hours after whole-body irradiation. Similarly, Kato _et al._ (129) concluded that x-irradiation, even in doses of 1400R, had no significant effect on the _in vitro_ oxidative metabolism of pentobarbital or meprobamate in rats. These investigators also measured enzyme activity only at 2 days after irradiation. Indeed, Figure 6 does show that 2 days after 1200R, no significant changes in the oxidative metabolism of aniline and aminopyrine are evident.

On the other hand, one must not overlook the possibility of a species difference in the radiosensitivity of hepatic detoxication systems in rats and mice. In the literature, a large majority of the studies concerned with radiation effects on drug-metabolizing enzyme systems in the liver used rats as the experimental animal. However, interpretations of data were often complicated by the fact that in rats, the response of drug-metabolizing enzyme systems to various unphysiological states is significantly sex dependent (103, 163).

Recent work by Kato *et al.* (110) has illustrated that there are clear species differences in the alterations of drug-metabolizing activity by liver microsomes under a variety of conditions. These workers have stressed the importance of determining the changes in hepatic enzyme activity produced by different unphysiological states in each separate species of animals before extrapolating data from one species to another.

A number of *in vitro* studies of several hepatic enzyme systems after whole-body irradiation have yielded data which was similar to the pattern of response that was observed in the experimental portion of this thesis. Hartiala *et al.* (135) showed that 400R caused an increase in the *in vitro* conjugation on the first post-irradiation day, followed by a decrease slightly below control level and later by an overcompensatory phase. Benes and Zicha (164) also observed that substrate induction of tryptophan oxygenase was stimulated 24 hours after x-irradiation with 1400R, followed later by a period of inhibition. After whole-body x-irradiation with 800R, the levels of alpha-hydroxy acid oxidase in mouse liver were significantly increased in the initial post-irradiation period followed by a subsequent decline in enzyme activity (165). Ichii *et al.* (130) reported that N-demethylase activity in rat hepatic microsomes decreased significantly on the fifth day after whole-body x-irradiation with 600R. A cyclic effect of whole-body gamma irradiation on the *in vitro* acetylation of sulfisoxazole in rat liver has also been noted in this

laboratory (166).

Several researchers have studied the effects of radiation on drug metabolism by measuring changes in the urinary excretion of metabolites after irradiation. Since it is currently accepted that drug metabolism is primarily associated with the enzyme systems in liver microsomes, some correlation should obviously exist between data obtained from the quantitation of urinary metabolites and from the *in vitro* methods of this present investigation which utilized isolated hepatic microsomal preparations. Thus, in agreement with the post-irradiation pattern in hepatic detoxication capacity which was presented in the experimental portion of this dissertation, Haley and Koste (134) noted a small rise in resorcinol-glucuronide excretion on the first day after irradiation with 600R, followed by a decrease in the excretion of the conjugate from the third day on. Noujaim *et al.* (166a) studied the effects of x-irradiation on the metabolism of amphetamine in rats and reported a cyclic pattern in metabolite excretion; i.e., an initial increase, followed by a depression by the seventh day and then an elevation above normal levels. After whole-body irradiation with doses of 1000R, the resulting impairment in hepatic detoxication processes have been reflected by a significant reduction in the excretion of conjugated sulfates (167) and glucuronides (168).

A number of studies into the interactive effects of radiation and drugs illustrated that modifications of

pharmacological activity may occur in the irradiated animal. Pharmacological response to a drug depends on a multitude of factors, including tissue distribution, availability and accessibility to effector sites, metabolic fate and excretion (136). Ionizing radiation is known to influence not only enzyme systems, but also membrane permeability, blood-brain barriers, endocrine activity, cellular morphology and excretory processes, depending on the quality and quantity of radiation (169). Thus, it would be imprudent to ascribe all the changes observed in pharmacologic activity to alterations in the hepatic microsomal detoxication systems. Indeed, the enhancement in onset of barbiturate hypnosis after cephalic x-irradiation has been association with radiation-induced increases in the permeability of the blood-brain barrier (136, 170). It has also been suggested that during irradiation, some humoral factor such as serotonin might be released which may then contribute to the prolongation of barbiturate hypnotic action (137, 170). Increased duration of barbiturate sleeping time in lethally irradiated (1,500R to 10,000R) monkeys has been ascribed to alterations of the blood-brain barrier permeability, decreased renal excretion and impairment in detoxication by liver enzymes (171).

The results which are reported in this present investigation concur with some of the changes reported in pharmacological responses of irradiated animals. The impairment in oxidative drug metabolism after 600R (Figure 4) may possibly explain why the barbiturate hypnosis was prolonged,

assay of hepatic p-nitrobenzoic acid reductase, the amounts of several components in the incubation media can influence the results to a very significant degree. Kinoshita *et al.* (174), employing the techniques of Hietbrink and DuBois (120), reported that chronic administration of DDT to male rats failed to produce enhanced hepatic reduction of p-nitrobenzoic acid, even though such treatment did stimulate the O- and N-demethylase activity in liver homogenates. Hart and Fouts (175) studied the effect of acute and chronic administration of DDT on several hepatic microsomal drug-metabolizing enzymes in rats and found a marked increase in the metabolism of p-nitrobenzoic acid after DDT treatment. In a subsequent investigation, Peters and Fouts (176) attempted to resolve the discrepancy between the two reports. The two assay procedures differed mainly in that Hart and Fouts (175) employed the 9,000 x g supernatant (equivalent to 333 mg of liver) as the source of enzymes and used 2.25 μmoles of NADP in the incubation mixtures, whereas Hietbrink and DuBois (120) used a 10% whole liver homogenate (equivalent to 50 mg of liver) and only 0.145 μmoles of NADP per incubation. Peters and Fouts (176) showed that if the amounts of NADP and liver were increased in the Hietbrink and DuBois incubation media, then a definite DDT stimulation of p-nitrobenzoic acid metabolism could be demonstrated. Thus, it is possible that the procedure employed by Hietbrink and DuBois (120) was not sufficiently sensitive to detect changes in p-nitrobenzoic acid metabolism after irradiation.

Chatterjee and McKee (177) have ascribed the inhibition of enzymatic activity in rat liver microsomes which resulted from lethal doses of whole-body x-irradiation to a reduction of food intake rather than to a definite effect of the radiation. It has been shown that starvation does alter the activity of hepatic drug-metabolizing enzymes in rats, with considerable sex differences being evident (103). Kato and Gillette (109) reported that after starvation, the activities of almost all the hepatic drug-metabolizing enzymes were enhanced in female rats, but that in male rats, the metabolism of zoxazolamine and p-aminobenzoic acid was unchanged; the activity of aminopyrine N-demethylase was impaired, and the hydroxylation of aniline was stimulated. However, Hietbrink and DuBois (120) and Nair (136), using starved rats as controls, concluded that changes observed after irradiation were specific radiation effects and were not due to prolonged fasting. Furthermore, Dixon _et al._ (108) reported that in mice, starvation caused a decrease in the activities of several oxidative drug-metabolizing enzymes, but that the hepatic reduction of p-nitrobenzoic acid or neoprontosil was not significantly affected. More recently, Kato _et al._ (110) showed that starvation did not alter the aminopyrine N-demethylase activity in liver microsomes of mice. In view of these observations, it is questionable whether the impairment noted in the hepatic detoxication systems of the irradiated mice in this presentation could be attributed to reduced food intake.

A number of investigators have approached the study of radiation damage to hepatic microsomal enzyme systems by measuring changes in various components of the electron transport system involved in drug metabolism. It has been well established that reduced nicotinamide adenine dinucleotide phosphate (NADPH) is necessary for the activity of oxidative and reductive metabolic pathways. Berliner et al. (178) introduced an hypothesis that ionizing radiation might produce a "coenzyme disease", i.e., a lack of NADPH or other cofactors necessary for the functioning of normal cells. However, Ichii and Kobayashi (179) measured the concentration of NADP and NADPH in rat liver at various intervals after 600R whole-body irradiation and observed no significant changes in the levels of these coenzymes.

Glucose-6-phosphate, together with glucose-6-phosphate dehydrogenase, serve to maintain NADP in a reduced form. DuBois, Raymund and Hietbrink (180) noted a small increase in hepatic glucose-6-phosphate after irradiation of young rats with 400R, but concluded that this change, alone, could not account for the radiation-induced defect in the microsomal oxidase reactions. These same researchers did not find any distinct alterations in the activity of glucose-6-phosphate dehydrogenase from 16 hours to 5 days after exposure of rats to 800R. Kivy-Rosenberg and co-workers (181), however, reported that 625R whole-body irradiation caused a small decrease in hepatic glucose-6-phosphate dehydrogenase activity. The data of this present study show that another very essential

component of the hepatic microsomal electron transport system can be severely impaired by exposure to ionizing radiation. Decreases in microsomal cytochrome P-450 after irradiation have probably resulted in the marked inhibition of hepatic drug-metabolizing capacity.

The data presented in Figures 3 to 6 and 8 to 11 indicate that the activities of oxidative and reductive drug-metabolizing enzymes are closely associated with levels of both microsomal protein and cytochrome P-450. The initial stimulation observed in drug-metabolizing enzyme activity after irradiation appeared to coincide mainly with the increases in microsomal protein content which were prominent up to third post-irradiation day. Several other investigators have also reported initial increases in hepatic microsomal protein after whole-body irradiation (182, 183, 184). It is possible that such increases in microsomal protein may represent an increase in enzyme synthesis as reflected by higher drug-metabolizing activity. It has been shown that high levels of microsomal protein can be attained not only through an enhancement in the rate of synthesis, but by a decrease in the rate of protein degradation as well (85, 86, 183). In the later post-irradiation stages, the microsomal protein content assumed control values, but the activities of drug-metabolizing enzymes were impaired when the levels of microsomal cytochrome P-450 were decreased. Similar observations have been reported in tumor-bearing rats (71, 72, 89) and in old rats (90) where no significant

changes were noted in hepatic microsomal protein, yet the activities of oxidative and reductive drug-metabolizing enzymes were decreased due to a marked decline in cytochrome P-450 levels. DuBois (124) observed that ethionine and puromycin were more efficient than x-irradiation in preventing the effects of phenobarbital enzyme induction which is characterized primarily by an increase in enzyme protein. In explanation, DuBois suggested that x-ray inhibition of microsomal enzyme activity must involve a different mechanism than the blockade of protein synthesis. Perhaps, as observed in this present study, the inhibitory effects of radiation may be due mainly to decreases in cytochrome P-450 rather than impairment of protein synthesis.

As indicated in the results, a very consistent response of mouse liver to ionizing radiation was the abrupt decline in drug-metabolizing enzyme activity, microsomal protein and cytochrome P-450 between the third and fifth post-irradiation days. If these biochemical changes were due to manifestations of radiation injury to mechanisms governing the synthesis and degradation of enzymes or coenzymes, then this type of damage would be evident at some period after radiation exposure, depending on the turnover rate of the enzyme molecules or coenzyme factors. In mouse liver, a half-life of 3.5 days and 2.8 days has been reported for microsomal protein (86) and NADPH-cytochrome c reductase (185) respectively. A half-life of 3 to 4 days for hepatic microsomal cytochrome P-450 has also been suggested (186). Thus it is

possible that the sharp drop observed in enzyme activity, microsomal protein and cytochrome P-450 content between the third and fifth post-irradiation days was associated with interference by the radiation, in the normal turnover of these components.

As outlined in the literature portion of this thesis, a considerable amount of evidence has been compiled to implicate cytochrome P-450 in the oxidative and reductive mechanisms of drug-metabolism. The activity of many drug-metabolizing enzymes has been shown to be related to the levels of microsomal cytochrome P-450 under a variety of unphysiological and pathological conditions as well as following the administration of enzyme inducers, inhibitors and hepatotoxic agents. In accordance with these observations, it is postulated that the changes in the activities of the drug-metabolizing enzymes following irradiation, as reported here, were due largely to the alterations in levels of cytochrome P-450. The activities of aniline p-hydroxylase, aminopyrine N-demethylase and p-nitrobenzoic acid reductase were noted to parallel the levels of cytochrome P-450 particularly after the third or fifth post-irradiation day.

An inadvertent exposure of a group of mice to cold, which resulted in a stimulation of hepatic drug-metabolizing systems, alerted the author to the fact that stress may play an important role in the response of these systems to radiation. As outlined in the literature survey, it has been established that various types of stress conditions can

influence the activity of hepatic detoxication systems. Driever and Bousquet (117) noted that actinomycin D could prevent the stress-induced stimulation of hepatic drug metabolism and postulated that the enhanced drug-metabolizing activity following limb-ligation was caused by increased synthesis of enzyme protein. Figures 7 and 12 indicate that cold-stress appeared to cause enzyme induction as characterized by elevated microsomal protein, cytochrome P-450 and drug-metabolizing enzyme activities. Similarities were noted in the reactions of the hepatic systems to cold or to low doses of gamma radiation during the first three days following exposure. This similarity of response suggested that the initial stimulation of the drug-metabolizing systems that was observed after irradiation may have been mediated by a stress reaction to the radiation. Such a proposal would be in agreement with Parke (187) who stated that exposure to ionizing radiation produces a typical stress response and like other stress conditions might be expected to result in the activation of the metabolism of foreign compounds.

VI. SUMMARY AND CONCLUSIONS

(1) Adult male mice were exposed to whole-body gamma irradiation ranging from 300R to 1200R and the effects on oxidative and reductive drug-metabolizing enzyme systems in the liver were measured at various post-irradiation times.

(2) Results from the *in vitro* studies indicated that exposure to ionizing radiation can alter the hepatic drug-metabolizing ability to a significant degree depending upon the radiation dosage and the time interval after exposure.

(3) After exposure to 300R, the activities of hepatic aniline p-hydroxylase, aminopyrine N-demethylase, and p-nitrobenzoic acid reductase increased on the third post-irradiation day, decreased to minimal values on the seventh day and subsequently regained control levels by the eleventh day.

(4) Following irradiation with 600R, a stimulation of both reductive and oxidative drug-metabolizing pathways was noted on the first and third days. After the third day, a decline to subnormal values was observed in the activities of aniline p-hydroxylase, aminopyrine N-demethylase and p-nitrobenzoic acid reductase. However, all three enzyme systems gradually assumed control levels again by the fifteenth to twenty-first day following 600R.

(5) The pattern of response of oxidative and reductive drug-detoxication enzymes in mouse liver to 950R was one of stimulation up to the third post-irradiation day followed later by severe inhibition with no recovery.

(6) Initially, 1200R of whole-body gamma irradiation caused a depression in the *in vitro* metabolism of aniline

and aminopyrine, and a slight elevation in nitro reduction. The small increases in enzyme activity on the third day were succeeded abruptly by a very significant impairment in the metabolism of all three substrates at subsequent time intervals.

(7) Increases in hepatic microsomal protein content were observed on the third day after exposure to 300R, and on the first and third days after 600R, 950R and 1200R. A return to normal or slightly subnormal levels occurred by the fifth post-irradiation day. Except for a decrease on day 7 after 950R, the protein levels in the liver microsomes of the irradiated mice did not deviate appreciably from control values during the remainder of the observation periods. The initial post-irradiation stimulation in the activities of drug-metabolizing enzymes was noted to coincide with the increases in microsomal protein content.

(8) Levels of microsomal cytochrome P-450 were elevated to various degrees on the third day after 300R, the first and third days after 600R, and on the first day following 950R. After exposure to 1200R, an initial depression in cytochrome P-450 content was observed. The amount of cytochrome P-450 in the liver microsomes from the irradiated mice was maximally depressed on the seventh day after 300R and on the eleventh day after 600R, but eventual recovery of P-450 levels to normal values was noted following these radiation doses. In the mice exposed to 950R or 1200R, severe depression of hepatic microsomal cytochrome P-450 continued after the third

post-irradiation day. The impairment in the activities of hepatic aniline p-hydroxylase, aminopyrine N-demethylase and p-nitrobenzoic acid reductase paralleled the low levels of cytochrome P-450 in the later post-irradiation stages.

(9) Adult male mice were stressed by exposure to cold. The activities of hepatic aniline p-hydroxylase, aminopyrine N-demethylase and p-nitrobenzoic acid reductase, as well as the content of microsomal protein and cytochrome P-450 were measured at the same post-exposure intervals as those which were used in the studies involving irradiated mice.

(10) The *in vitro* metabolism of all three drugs was stimulated after cold stress. Marked increases in hepatic aniline p-hydroxylase activity were noted on the first and fifth day after exposure. Aminopyrine N-demethylase activity was significantly enhanced on the first and third day, while p-nitrobenzoic acid reductase was elevated on the first, third and fifth days. Increases in microsomal protein were evident primarily on the first day after stress. Levels of microsomal cytochrome P-450 were significantly elevated on day 3 and 5. Similarities were noted in the patterns of response exhibited by these hepatic detoxication systems to cold and to lower levels of irradiation during the early stages after exposure.

(11) It was concluded, from the data obtained, that ionizing radiation, even in sublethal doses, causes injury to the hepatic drug detoxication systems. Whereas acute cold-stress effected only a stimulation in post-exposure drug-

metabolizing enzyme activity, irradiation, in lower doses, produced an initial stimulation followed by varying periods of significant inhibition. Higher doses of gamma irradiation resulted in irreparable damage to the activities of some drug-metabolizing enzymes by non-reversible depression of microsomal cytochrome P-450.

(12) Measurement of microsomal protein alone, is not a satisfactory indication of the extent of radiation impairment to hepatic drug metabolism. Early post-irradiation increases in microsomal protein may be related to a radiation-induced stress reaction.

(13) Determination of microsomal cytochrome P-450 levels was shown to be invaluable in assessing radiation injury to the drug-metabolizing activity of the liver, particularly during later post-irradiation periods. Estimation of both, microsomal protein and cytochrome P-450 were useful in accounting for the various changes which were observed in the hepatic drug-metabolizing activities after whole-body irradiation.

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VIII. APPENDIX

Figure I

Standard Curve for p-Aminophenol Recovered from Incubation Media

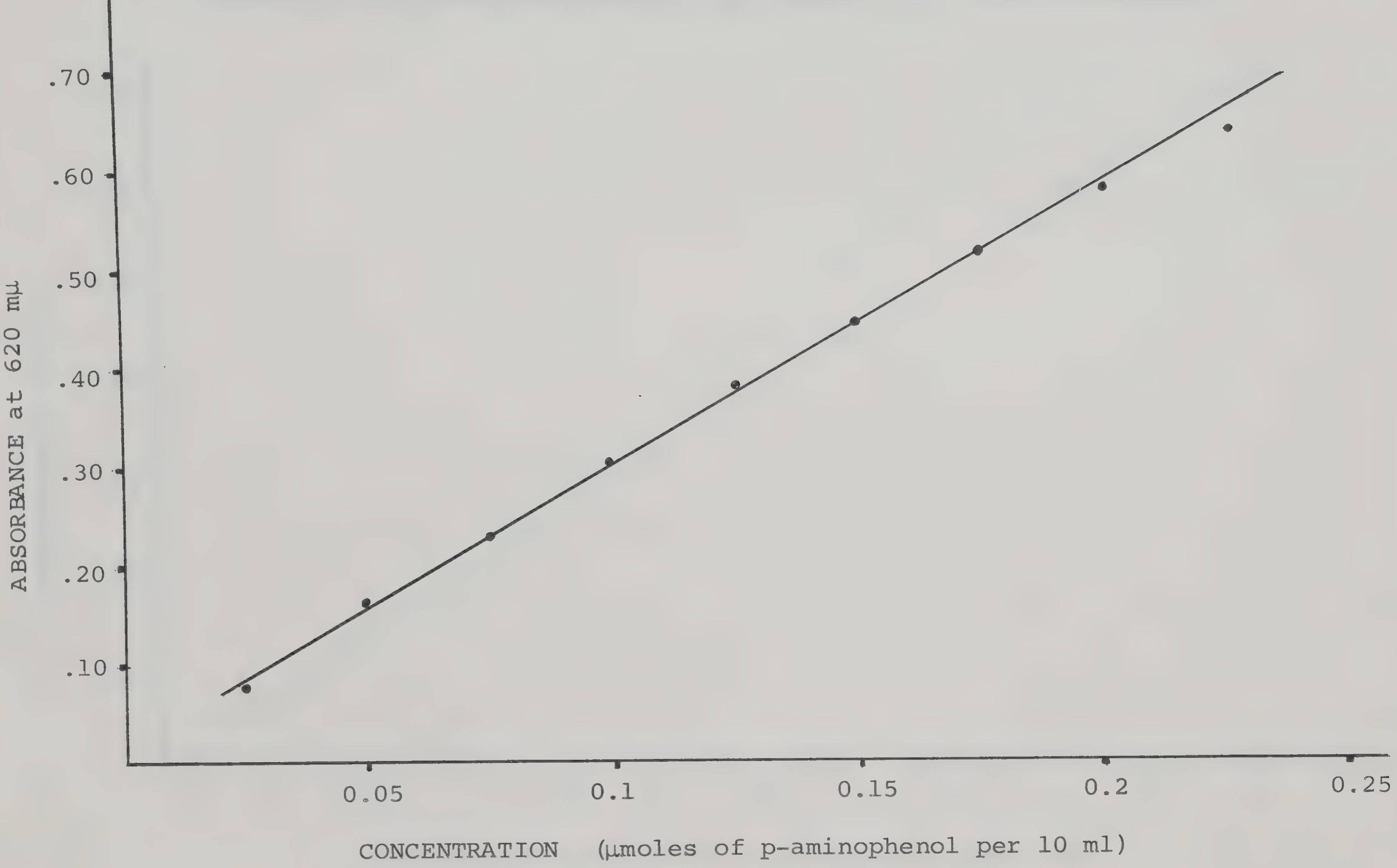


Figure II

Standard Curve for Formaldehyde Recovered from Incubation Media

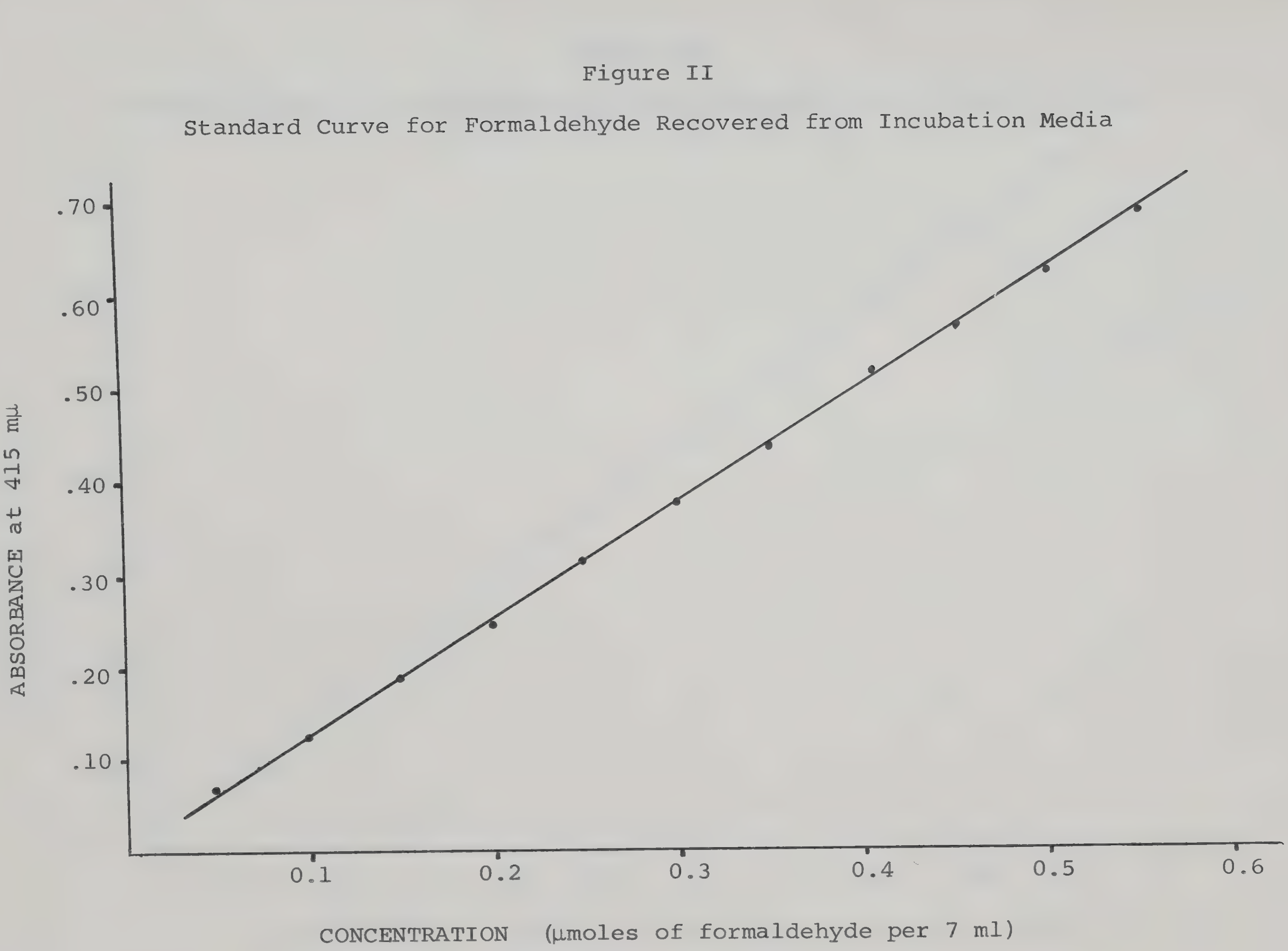


Figure III

Standard Curve for p-Aminobenzoic Acid Recovered from Incubation Media

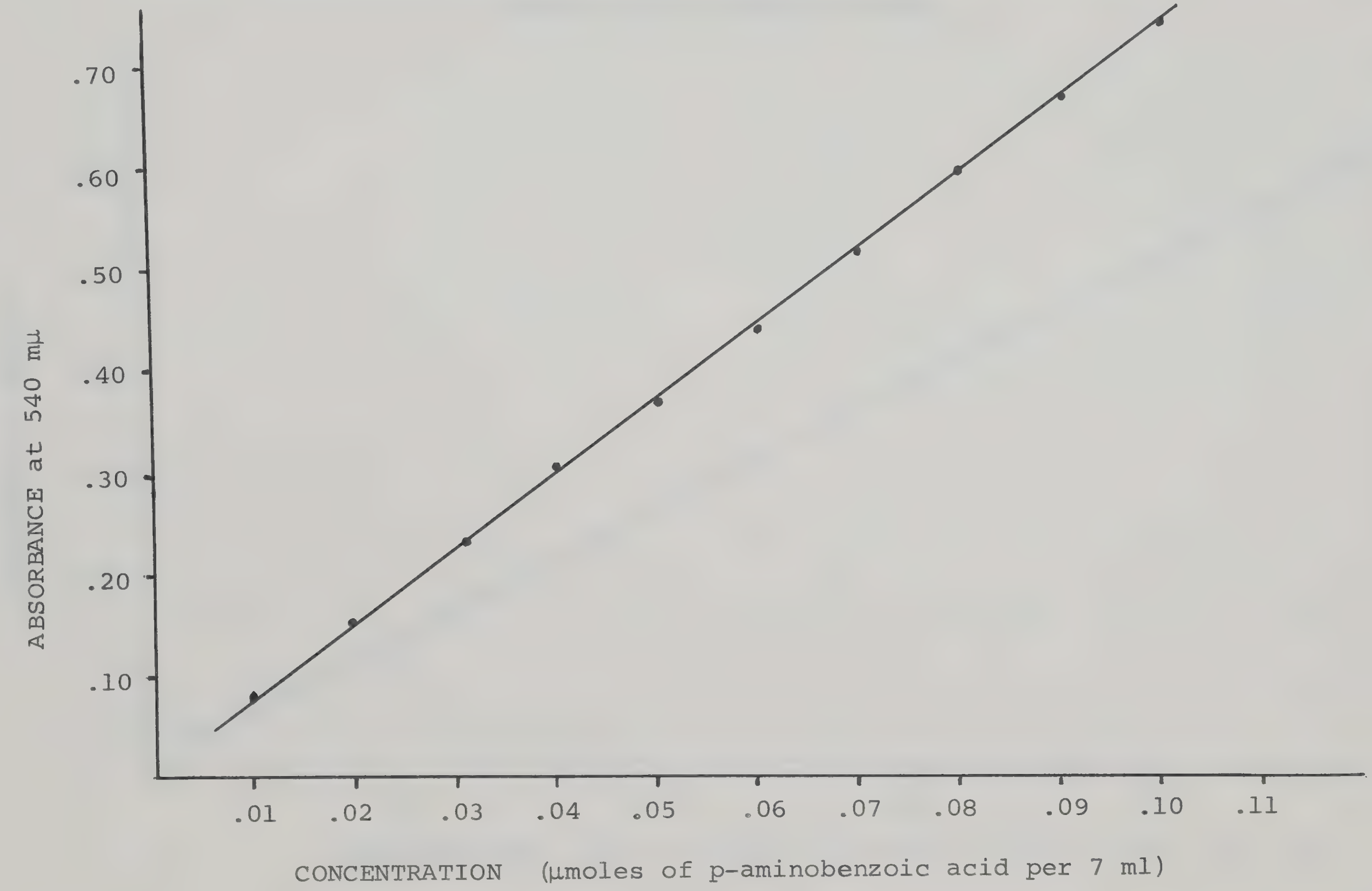


Figure IV

Standard Curve for Determination of Microsomal Protein
Content by the Lowry Method

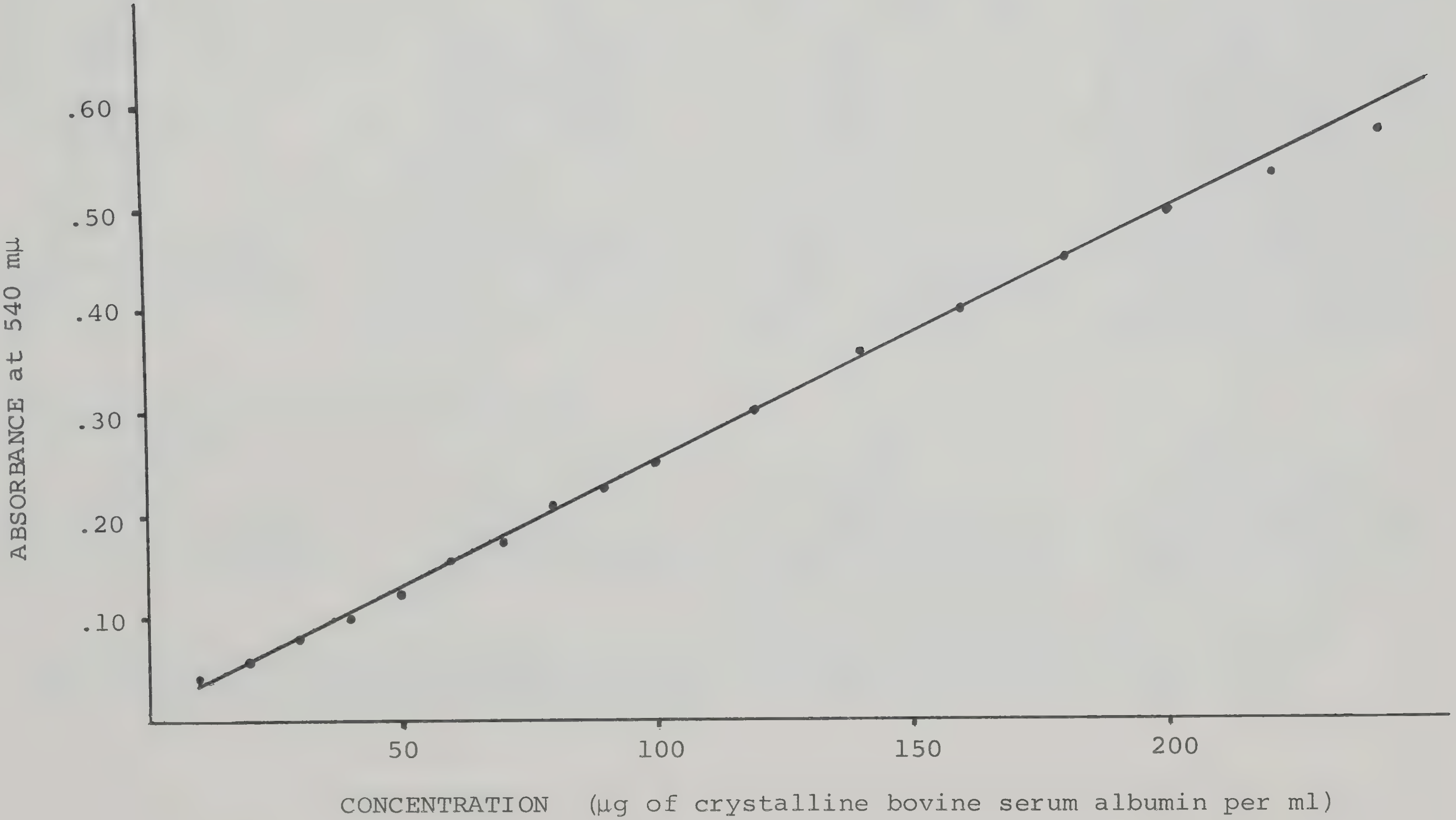
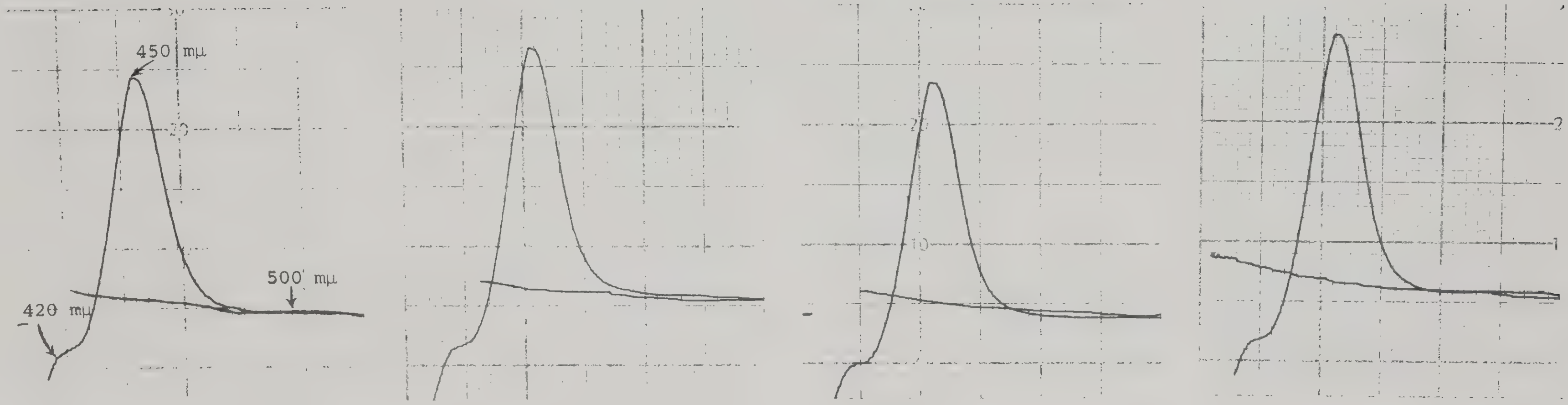


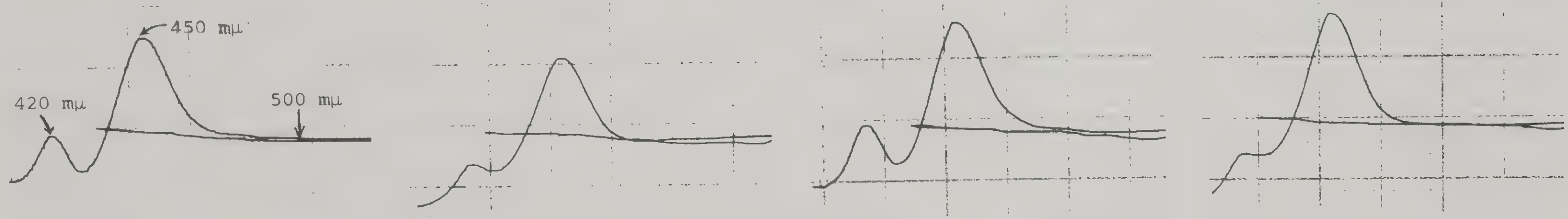
Figure V

Cytochrome P-450 Spectra

CONTROL MICE:



FIVE DAYS AFTER WHOLE-BODY IRRADIATION WITH 1,200R:



Spectra illustrating the carbon monoxide complex of dithionite-reduced liver microsomes from control and irradiated mice. Microsomal protein content was adjusted to 2 mg per ml. The secondary peak at 420 mμ in the spectra from the irradiated mice may be due to hemoglobin contamination.

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